An Investigation into the Intracellular Mechanisms Involved in Prostaglandin Production by the Guinea-Pig Uterus and Placenta.

By

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ABSTRACT OF THESIS

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Title of Thesis AN INVESTIGATION INTO THE INTRACELLULAR MECHANISMS INVOLVED IN PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG UTERUS AND PLACENTA.

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This thesis has studied intracellular mechanisms involved in prostaglandin (PG) synthesis by and release from the guinea-pig uterus and placenta. Adenosine, ATP and its analogues stimulated prostaglandin output (particularly $PGF_{2\alpha}$) from the superfused guinea-pig uterus, and from the endometrium and myometrium cultured for 24 h. Adenosine and ATP-induced $PGF_{2\alpha}$ output from the superfused guinea-pig uterus was inhibited by the adenosine (A) receptor antagonist, 8-sulphophenyltheophylline and the P2 purinoceptor antagonist, suramin, respectively. Adenosine and ATP-induced 6-keto-$PGF_{1\alpha}$ output from the superfused guinea-pig uterus was unaffected by 8-sulphophenyltheophylline and suramin respectively. Therefore, adenosine and ATP-induced increases in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus appear to be receptor mediated responses, while the mechanisms involved in 6-keto-$PGF_{1\alpha}$ production remain unclear.

PG output, particularly $PGF_{2\alpha}$, from the guinea-pig placenta and sub-placenta cultured for 24 h increased significantly between days 22 and 29 of pregnancy. Metabolism of $PGF_{2\alpha}$ by the placenta also increased between these two days suggesting that increased PG output was not due to decreased metabolism. Indomethacin, a non-selective inhibitor of PGHS, and NS-398 a PGHS-2 selective inhibitor, reduced PG output from day 22 and day 29 guinea-pig placenta and sub-placenta after 24 h of culture. PGHS-2 appears to be the predominant enzyme responsible for $PGF_{2\alpha}$ synthesis by day 22 and day 29 guinea-pig placenta and sub-placenta, while both isoforms of the PGHS enzyme are involved in PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis by day 22 and day 29 guinea-pig placenta and sub-placenta.

The outputs of $PGF_{2\alpha}$, and PGE_2 from the placenta and sub-placenta and 6-keto-$PGF_{1\alpha}$ output from day 29 guinea-pig placenta in culture were reduced by the inclusion of cycloheximide, puromycin and actinomycin D (protein synthesis inhibitors) in the culture medium. This suggests that the production of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ is dependent on protein synthesis.

The presence of calcium-depleted medium inhibited 6-keto-$PGF_{1\alpha}$ output from day 29 guinea-pig placenta after 24 h of culture, but had no effect on $PGF_{2\alpha}$ and PGE_2 outputs. This suggests that 6-keto-$PGF_{1\alpha}$ production is dependent on extracellular calcium, while $PGF_{2\alpha}$ and PGE_2 production by day 29 placenta is not. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 29 guinea-pig sub-placenta were inhibited in the presence of calcium-depleted medium, which suggests that extracellular calcium is a requirement for their synthesis. The inclusion of EGTA (a calcium chelator), TMB-8 (a calcium antagonist), TFP and W-7 (calmodulin antagonists) in tissue culture medium, inhibited 6-keto-$PGF_{1\alpha}$ output from the placenta after 24 h of culture. Therefore, 6-keto-$PGF_{1\alpha}$ output from day 29 guinea-pig placenta is dependent on intracellular calcium and calmodulin for its synthesis. $PGF_{2\alpha}$ and PGE_2 production by day 29 guinea-pig placenta was also inhibited by TMB-8 and are dependent on intracellular calcium for their synthesis. TFP and W-7 had no inhibitory effect on $PGF_{2\alpha}$ and PGE_2 outputs from the guinea-pig placenta after 24 h of culture. Therefore, calmodulin does not appear to be necessary for $PGF_{2\alpha}$ and PGE_2 production by the placenta. PG production by the guinea-pig sub-placenta was inhibited by the calcium antagonist TMB-8 but was not inhibited by the calmodulin antagonists TFP and W-7. Therefore, $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by day 29 guinea-pig sub-placenta is dependent on intracellular calcium but not on calmodulin. PG output from day 29 placenta and sub-placenta was inhibited by nifedipine and verapamil (calcium channel blockers) which suggests that $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production requires calcium entry via voltage-dependent calcium channels. Inclusion of TPA (a PKC activator) in the culture medium demonstrated that PKC is not a requirement for $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ synthesis by day 29 guinea-pig placenta or sub-placenta since TPA had no effect on PG output.

Luteinising hormone releasing hormone (LHRH) appeared to have opposing actions on $PGF_{2\alpha}$ and PGE_2 outputs after 24 h of culture, inhibiting $PGF_{2\alpha}$ and PGE_2 outputs from day 29 guinea-pig placenta and stimulating $PGF_{2\alpha}$ and PGE_2 output from the sub-placenta. LHRH had little effect on 6-keto-$PGF_{1\alpha}$ output from the guinea-pig sub-placenta and had opposing actions on 6-keto-$PGF_{1\alpha}$ output from the placenta, inhibiting 6-keto-$PGF_{1\alpha}$ output after 2 h and increasing it after 24 h of culture.

This work has shown that the mechanisms involved in PG production by the guinea-pig uterus and placenta are complex. These include the synthesis and availability of PLA_2, PGHS and calcium (extracellular and intracellular).

In accordance with the requirements of regulation 3.4.7 this thesis has been composed by myself and the work presented herein is my own.

Heather Aitken

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ATP	Adenosine 5'-Triphosphate
DARS	Donkey anti-rabbit serum
EGTA	Ethylene glycerol-bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid
GnRH	Gonadotrophin Releasing Hormone
IP_3	Inositol-1, 4, 5-triphosphate
MAPK	Mitogen-Activated Protein Kinase
NRS	Normal rabbit serum
NS-398	N(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide
PG	Prostaglandin
PGFM	13, 14-dihydro-15-keto-$PGF_{2\alpha}$
PGHS-1	Prostaglandin H Synthase-1
PGHS-2	Prostaglandin H Synthase-2
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4, 5-biphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
TFP	Trifluoperazine
TMB-8	8-(N, N-diethylamino)-octyl-3, 4, 5-trimethoxybenzoate hydrochloride
TPA	Phorbol 12-myristate-13-acetate
UTP	Uridine Triphosphate
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide

CONTENTS

SECTION TWO

SECTION ONE

1 GENERAL INTRODUCTION

1.1 PROSTAGLANDINS

1.1.1 The History of Prostaglandins

The discovery of prostaglandins (PGs) is attributed to American gynaecologists Kurzrok & Lieb (1930). Kurzrok & Lieb (1930) demonstrated that fresh human semen contained a substance which contracted and relaxed human uterine smooth muscle *in vitro*. In 1935, von Euler and Goldblatt independently observed that extracts from the prostatic and related glands of human, monkey, sheep and goat, and extracts from sheep seminal vesicles caused contraction of smooth muscle. Discovery of this substance in the human and sheep prostate gland prompted von Euler to suggest the name prostaglandin (von Euler, 1939). Despite their name prostaglandins were later discovered to be synthesized by the seminal vesicles and not by the prostate gland (Eliasson, 1959).

Limited techniques restricted analysis but von Euler (see Moore, 1985) was the first to attempt extraction of prostaglandin. He demonstrated that prostaglandin was soluble in both water and organic solvents, was destroyed at extremes of pH, lacked nitrogen and was an unsaturated fatty acid. The chemical nature of prostaglandin was later confirmed by Bergström (see Moore, 1985). Bergström also reported that seminal fluid extract contained more than one prostaglandin (see Moore, 1985).

It was a decade later before prostaglandins were isolated and recognised as a family of compounds. In 1957, Bergström & Sjovall published the structure of

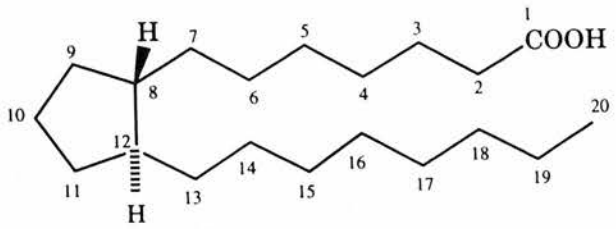
prostaglandin 'factor' (later shortened to PGF) extracted from sheep seminal vesicles. Prostaglandin E was also isolated from sheep vesicular glands (Bergström & Sjovall, 1957). The structures of $PGF_{1\alpha}$ and PGE_1 were later confirmed by Bergström *et al.* (1963). Subsequently more prostaglandins were discovered and named alphabetically i.e. PGA, B, C and D (see Fig. 1.1.2.1).

In the 1970s, cyclic endoperoxide prostaglandins (i.e. PGG_2 and PGH_2) were found to be intermediates in prostaglandin synthesis (Hamberg & Samuelsson, 1973) (see Fig. 1.1.2.1). In addition two other compounds were discovered i.e. thromboxane A_2 (Hamberg *et al.*, 1975) and prostacyclin (PGI_2) (Moncada & Vane, 1979). Also, Vane (1971) demonstrated that aspirin-like drugs inhibited prostaglandin synthesis. Also in the 1970s key roles of prostaglandins in reproduction became evident (see Poyser, 1981), but there is still much to be learned about their synthesis, catabolism and mechanism of action in reproductive tissues.

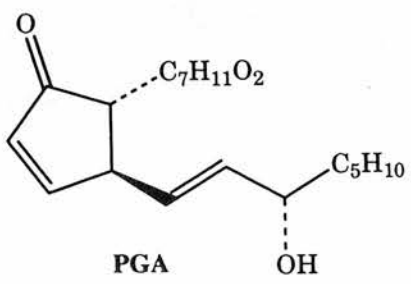
1.1.2 Structure, Chemistry, Biosynthesis & Metabolism

Prostaglandins (PGs), thromboxanes and leukotrienes are collectively known as eicosanoids. They influence and are produced by all nucleated cells in the body (Christ & van Dorp, 1972). PGs are not stored in the tissues but are synthesised and released from the cell as required (Piper & Vane, 1971). They have a short half-life and are rapidly metabolised especially in the lungs, liver, kidneys and the placenta.

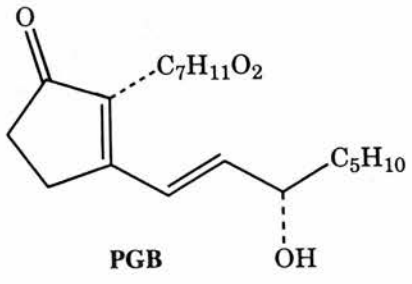
Prostaglandins are 20 carbon-containing, unsaturated fatty acids consisting of a cyclopentane ring and two side chains of 7 and 8 carbons. They are classified according to which group is attached to the cyclopentane ring and to the side chain. PGs are also sub-divided into three groups depending on the number of double bonds



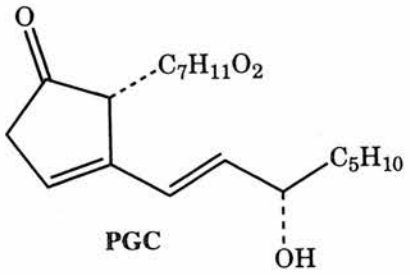
PROSTANOIC ACID



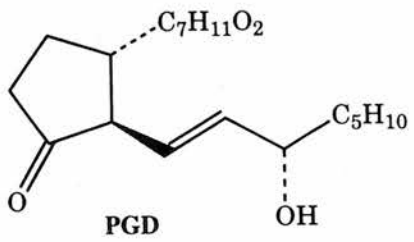
PGA



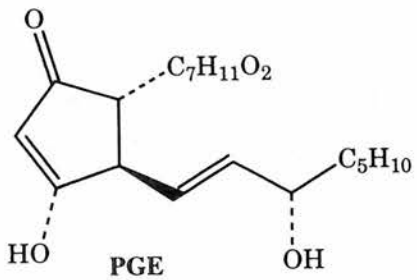
PGB



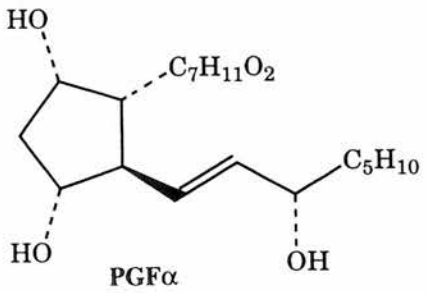
PGC



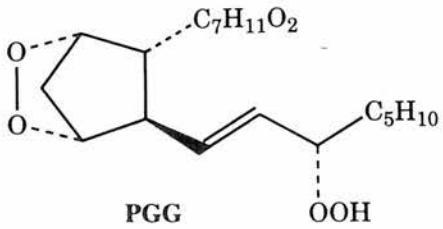
PGD



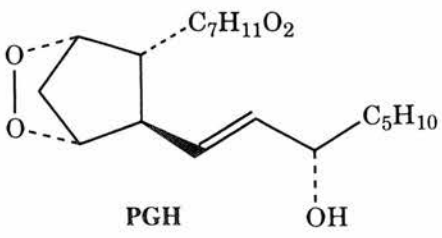
PGE



PGFα



PGG



PGH

Figure 1.1.2.1 Structure of prostanoic acid and structural differences between prostaglandin A to H. The structure for prostaglandin (PGI_2) is shown in Figure 1.1.2.2.

present. The majority of PGs in mammalian tissues have 2 double bonds between carbons 5 and 6 and carbons 13 and 14, signified by the 2 subscript as in $PGF_{2\alpha}$. The subscript α signifies the stereochemical configuration of the hydroxyl group at C9.

It was reasoned from the structure of prostaglandins that they were derived from an unsaturated fatty acid. Bergström *et al.* (1964) and van Dorp *et al.* (1964) demonstrated the conversion of radioactive arachidonic acid (cis-5, 8, 11, 14-eicosatetraenoic acid; arachidonic acid)) to PGE_2 in sheep seminal vesicle homogenates. Arachidonic acid was also observed to be converted into $PGF_{2\alpha}$ by guinea-pig lung homogenates (Ånggard & Samuelsson, 1965).

Prostaglandins are synthesised from fatty acids that make up the structural part of the plasma membrane. The specific PG or leukotriene produced depends on which fatty acid is in the membrane and which enzymes are present in the cell. The main precursor of eicosanoids, and particularly prostaglandins of the 2-series, is arachidonic acid, which is taken in via the diet or is enzymatically converted from linoleic acid, which is also taken in through the diet. PG precursors (e.g. arachidonic acid) are found bound to phospholipids and require phospholipases to free them. Phospholipids have a backbone of 3 carbon atoms on which saturated fatty acids, unsaturated fatty acids and bases are attached. For example, phosphatidylinositol exists in cell membranes as a glycerol backbone with a saturated fatty acid attached at position 1, an unsaturated fatty acid (e.g. arachidonic acid) at position 2, and inositol through a phosphate group at position 3. Arachidonic acid is generally the most abundant of unsaturated fatty acids in biological tissues.

The mobilisation of arachidonic acid from phospholipids is attributed to the action of phospholipase A_2 (PLA_2). PLA_2 attacks position 2 and releases unsaturated fatty acid.

The free arachidonic acid is then converted to PGH_2 by the action of prostaglandin H synthase (PGHS or COX). PGHS has two different enzymatic activities. This first step is the cyclooxygenase component of the reaction, which involves the conversion of arachidonic acid to the cyclic endoperoxide PGG_2. The cyclooxygenase reaction is followed by the peroxidase reaction and the rapid conversion of PGG_2 to PGH_2 (see Fig. 1.1.2.2). The final form of prostaglandin produced is dependent upon the cell type and the enzymes contained therein (Samuelsson *et al.*, 1978). Recently it has been discovered that there are two forms of PGHS, named PGHS-1 (or COX-1) (a constitutive enzyme) and PGHS-2 (or COX-2) (an inducible enzyme) (Hedin *et al.*, 1987; Wong & Richards, 1991).

The major site of PG inactivation (particularly of PGE_2 and $PGF_{2\alpha}$) is in the lungs, although PGs are rapidly metabolised in most tissues including the placenta. The initial step of PG catabolism is their uptake from the extracellular space across the plasma membrane; they then pass into the cytoplasm which contains degradative enzymes. The first enzyme responsible for the biological inactivation of PGs is 15-hydroxyprostaglandin dehydrogenase (15-PGDH) of which there are 2 types, namely Type I and Type II (see Hansen, 1976). This enzyme converts, for example, $PGF_{2\alpha}$ into 15-keto-$PGF_{2\alpha}$. This compound is then metabolised by 13, 14-prostaglandin dehydrogenase into 13, 14-dihydro-15-keto-$PGF_{2\alpha}$. This is the main circulatory metabolite. This compound is further metabolised by enzymes in the liver into dinor- and tetranor compounds. Dienoic acids may also be produced. The metabolites are excreted in the urine.

Prostaglandins regulate physiological and pathological processes in all body systems,

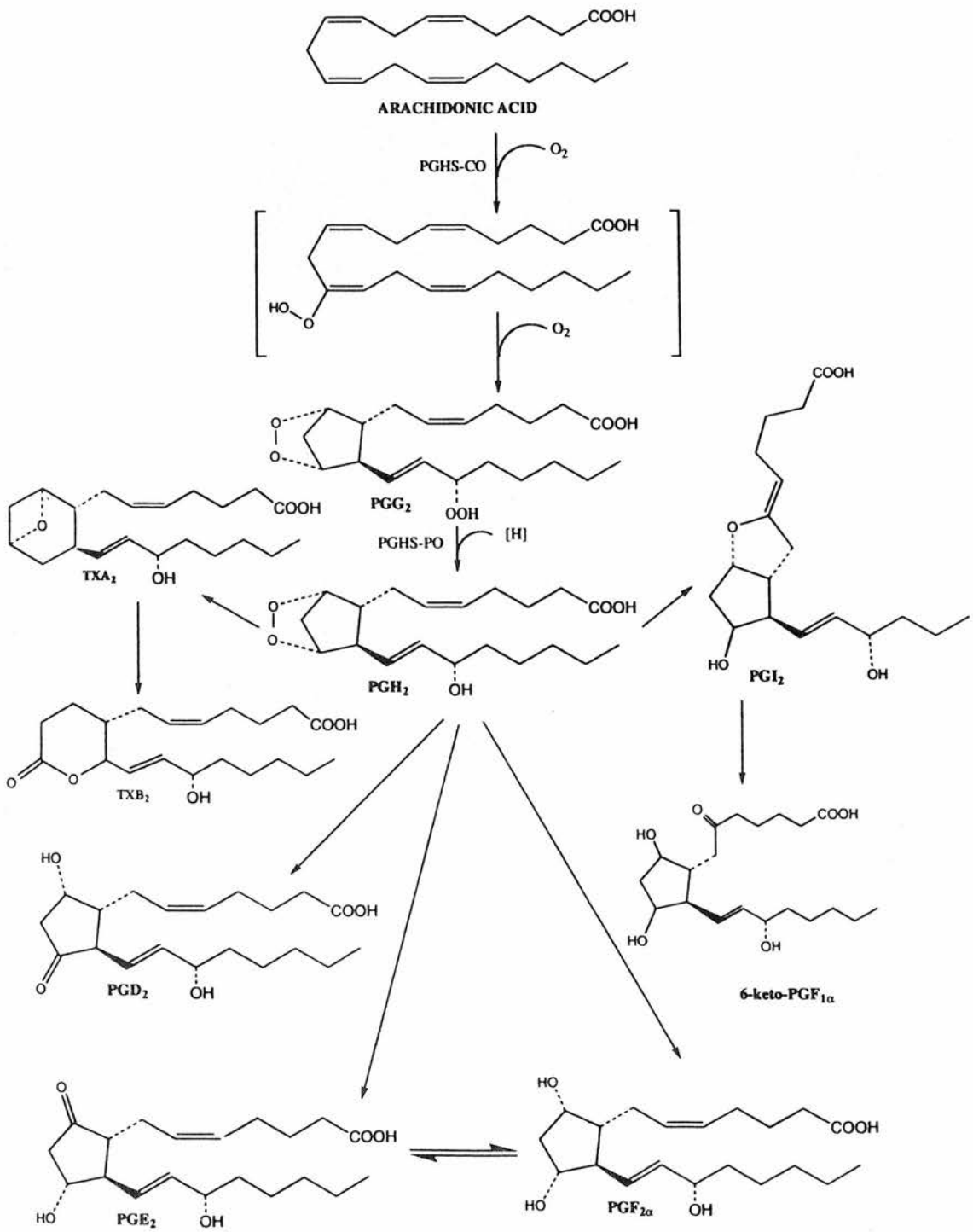


Figure 1.1.2.2. Major pathways of biosynthesis of prostaglandins (PG) and thromboxanes of the '2' series. PGHS-CO: cyclooxygenase component of prostaglandin H synthase enzyme complex. PGHS-PO: peroxidase component of prostaglandin H synthase enzyme complex. 12-L-hydroxy-5,8,10-heptadecatrienoic (HHT) and malondialdehyde (MDA) may also be formed from PGH_2.

including the reproductive processes. Therefore, the mechanisms controlling PG production by and action on reproductive tissues have been extensively studied.

1.1.3 Prostaglandins and Reproduction

In reproduction the stable prostaglandins, $PGF_{2\alpha}$ and PGE_2, appear to be the most important prostaglandins and seem to be necessary for several reproductive processes. Gonadotrophin releasing hormone (GnRH) is released in a pulsatile manner from peptidergic neurons originating in the hypothalamus and stimulates release of follicle stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary gland. These hormones promote growth of follicles within the ovaries and ovulation, respectively. There is much evidence that PGs are involved in the post-ovulatory surge of LH. Administration of PGs increases plasma concentrations of LH in sheep (Carlson *et al.*, 1973), and in pro-oestrous rats and ovarectomized rats (Tsafriri *et al.*, 1973; Kimball *et al.*, 1979). Similar stimulatory effects of PGs have been observed in other mammalian species including cattle (Louis *et al.*, 1974), rabbit (Carlson *et al.*, 1977a), hamster (Saksena *et al.*, 1974) and monkey (Carlson *et al.*, 1977b). PG synthesis inhibitors delay the LH surge at the start of the cycle in pro-oestrous rats (Brown & Poyser, 1984), which provides further evidence that PGs are involved in LH release. The stimulatory action appears to be directly on the gonadotrophin releasing hormone (GnRH) neurons, as there is no effect when PGE_2 is injected into the anterior pituitary gland while injection into the third ventricle stimulates LH release (Harms *et al.*, 1974).

PGs also appear to be involved in ovulation. The ovarian concentrations of PGs increase prior to ovulation in many species including sheep, pig, rat, rabbit and horse

(see Poyser, 1992). This is particularly true regarding the concentrations of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ in follicles destined to ovulate. Indomethacin has been observed to prevent ovulation in sheep, cattle, pigs, rats, mice, monkeys and women (see Poyser, 1992). Ovulation is suggested to occur due to thinning of the follicular wall through activation of collagenase by PG stimulation (see Poyser, 1995).

Luteolysis is the regression of the corpus luteum, the structure in the ovary that secretes progesterone. It was first suggested by Loeb (1923) that the uterus may secrete a luteolytic hormone, but it is only in the last thirty years that this hormone has been identified as $PGF_{2\alpha}$ in many non-primate mammalian species (see Poyser, 1992). $PGF_{2\alpha}$ may reach the ovaries through a counter-current mechanism between the ovarian artery and the utero-ovarian vein and/or the uterine lymph vessels (see Poyser, 1995). In the ovary, $PGF_{2\alpha}$ combines with specific receptors (FP) leading to functional and structural luteolysis. If a successful fertilisation occurs, then this process has to be prevented. In several species (e.g. sheep and cow) an interferon-α (now called interferon-τ) is responsible for inhibiting $PGF_{2\alpha}$ production by the uterus during early pregnancy (see Poyser, 1995).

Measurement of the $PGF_{2\alpha}$ metabolite, 5α, 7α-dihydroxy-11-ketotetranor-prostanoic acid, in guinea-pig urine showed that $PGF_{2\alpha}$ production by the early pregnant guinea-pig uterus is suppressed (Granström & Kindahl, 1976). This is due to reduced $PGF_{2\alpha}$ output from the uterus (see Poyser, 1995). The factor responsible for suppressing $PGF_{2\alpha}$ synthesis by the early pregnant guinea-pig uterus is not known, but it is not an interferon (Leckie & Poyser, 1990). The concentration of the $PGF_{2\alpha}$ metabolite in the guinea-pig urine rapidly increases again at approximately day 25 of gestation and

remains high as pregnancy proceeds. Therefore, from day 25 there must be increased $PGF_{2\alpha}$ production by the pregnant guinea-pig uterus or its contents. Norman & Poyser (1998b) observed that $PGF_{2\alpha}$ output from the guinea-pig placenta increases 15-fold between days 22 and 36 of pregnancy and speculated that this increase in $PGF_{2\alpha}$ output may account for the increase in concentration of the $PGF_{2\alpha}$ metabolite observed in guinea-pig urine from day 25 of pregnancy.

1.1.4 The Placenta and Prostaglandins

The placenta is a specialised organ that develops in the pregnant female. It is very important to the growing fetus and contains fetal and maternal components. The placenta functions by exchanging nutrients and secreting hormones that help to maintain pregnancy.

The guinea-pig placenta resembles the human placenta in that it is discoid and haemochorial in structure and is therefore a potential model for studies of human placental function. (Egund & Carter, 1974). In addition implantation is interstitial as in man. Figures 1.1.4.1-3 show photographs taken from a day 29 guinea-pig, illustrating the uterine horns, conceptuses, and dissection of the placentae. The guinea-pig placenta consists of two parts, the main placenta (chorio-allantoic placenta), within which fetal-maternal exchanges occur and, the sub-placenta that interfaces with the maternal tissues. The sub-placenta appeared as a gelatinous ovoid structure, greyish in colour, while the chorio-allantoic placenta appeared dark red in colour (Fig. 1.1.4.3). The main placenta is separated from the sub-placenta by a plane of fetal mesenchyme (Fig. 1.1.4.3). Recent work has helped to define the

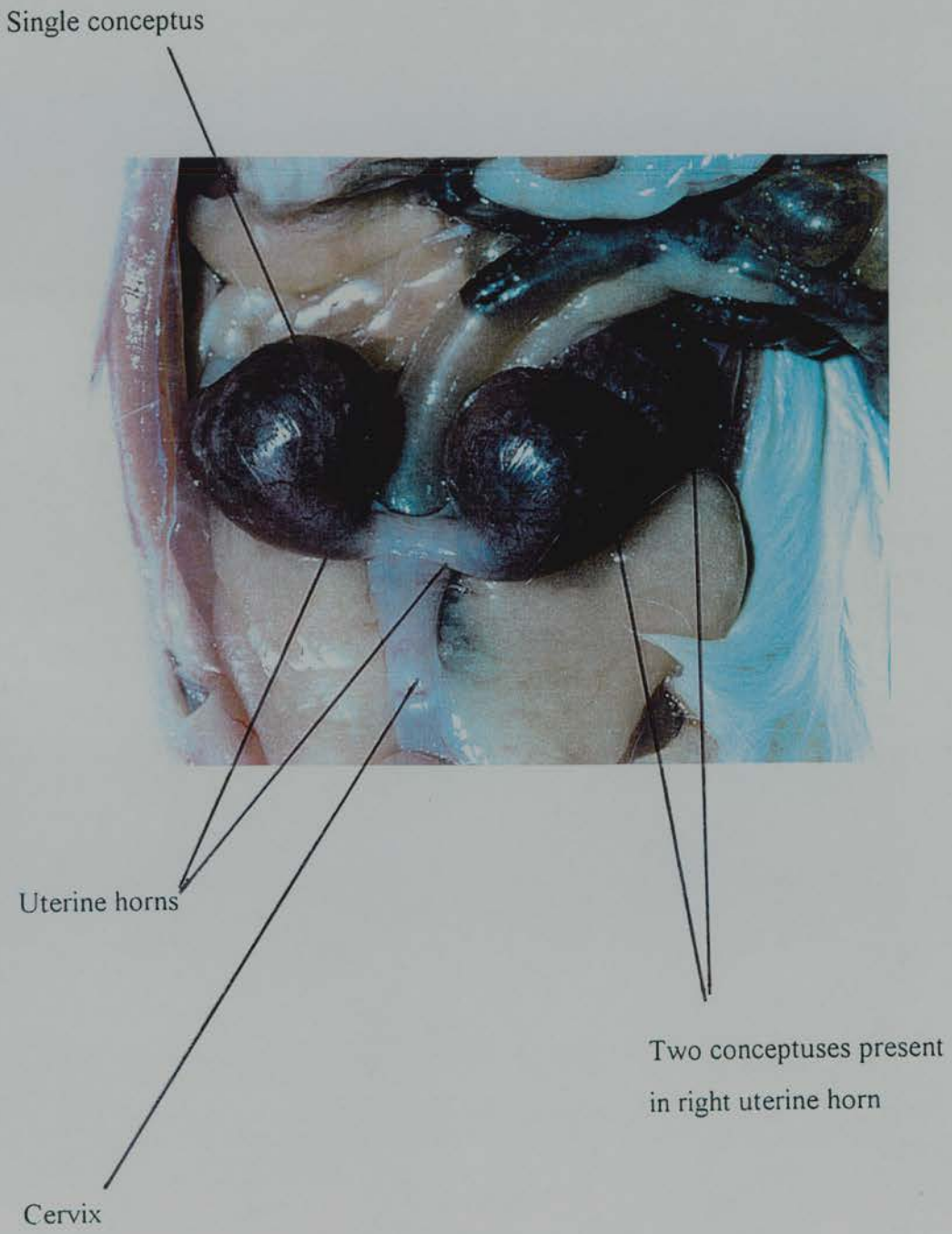


Figure 1.1.4.1 Photograph of ventral dissection of day 29 pregnant guinea-pig demonstrating position of cervix, uterine horns and conceptuses.

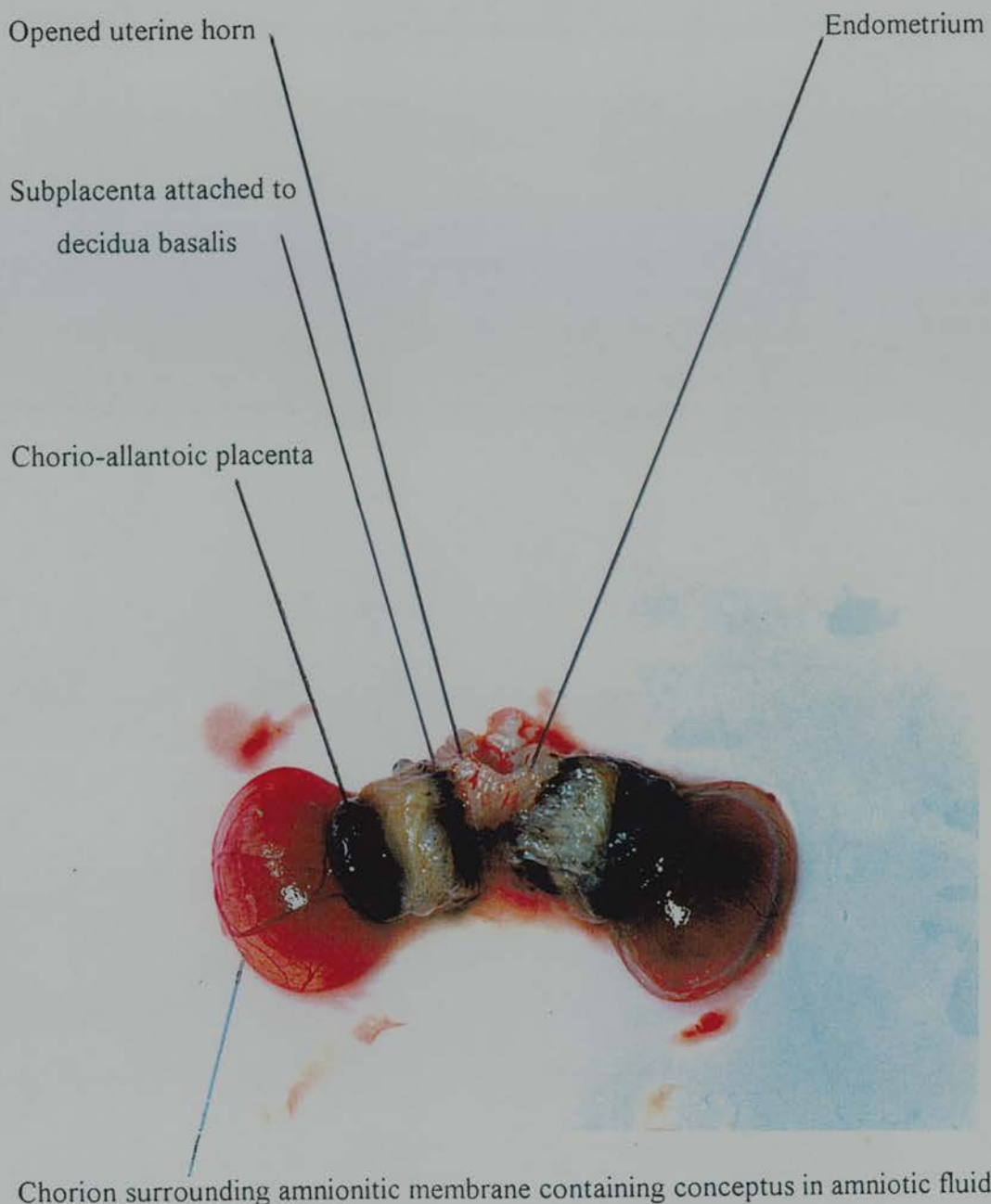


Figure 1.1.4.2 Photograph demonstrating dissection of day 29 pregnant guinea-pig with single uterine horn opened, revealing two conceptuses attached to endometrium by sub-placenta.

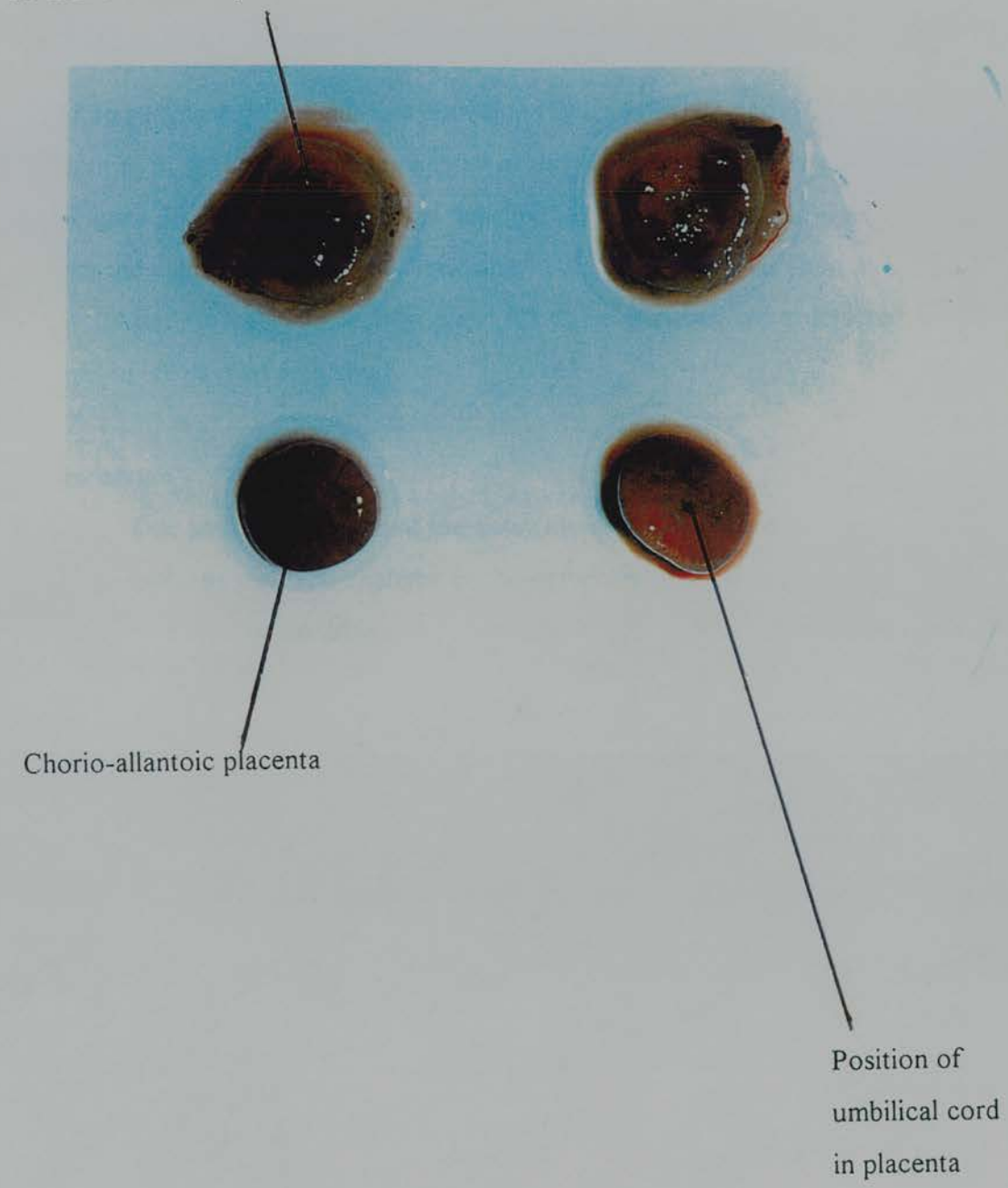


Figure 1.1.4.3 Photograph of dissection of day 29 pregnant guinea-pig. The two placentae, namely the sub-placenta and the chorio-allantoic placenta, are shown.

epithelial and mesenchymal cell types of the guinea-pig placenta (Carter *et al.*, 1998).

All intrauterine tissues, including the placenta, have the potential for PG production but it is not clear which tissue makes the most important contribution or how the synthetic pathways in different tissues are co-ordinated to obtain the required level of PG in the appropriate place and amount (Mitchell, 1986). Proposed functions for increased PG synthesis by the uterus and/or its contents during pregnancy include regulation of uterine contractions, ripening of the cervix, regulation of the patency of the ductus arteriosus, luteolysis and regulation of utero-placental haemodynamics (Hoedemaker *et al.*, 1991). They may also play a role in the regulation of blood flow in the utero-fetal unit (Moussard *et al.*, 1986).

It has been observed that the placenta is a major site of PG biosynthesis during pregnancy (Keirse, 1979). The fetal portion of the placenta (chorio-allantoic placenta) appears to have a higher capacity for prostanoid synthesis compared to the sub-placenta (maternal placenta) (Lytton & Poyser, 1982) or to other intrauterine tissues (Mitchell & Flint, 1977; Wimsatt *et al.*, 1993). PG levels in the rabbit fetal placenta are seven times higher than in the maternal placenta (Lytton & Poyser, 1982).

The guinea-pig placenta is an important source of endogenous PGs and may contribute to the augmented intra-uterine availability of prostanoids near parturition (Moussard *et al.*, 1986). Csapo *et al.* (1981) reported a sustained increase in $PGF_{2\alpha}$ output from the guinea-pig placenta from day 30 of pregnancy, and it has also been observed that the guinea-pig placenta has a decreased capacity for PG metabolism in late gestation (Moussard *et al.*, 1986). Schellenberg & Kirby (1997) also

demonstrated that PG output from the guinea-pig placenta tends to increase as pregnancy proceeds. Also mentioned earlier, recent studies have shown that $PGF_{2\alpha}$ output from the guinea-pig placenta increases 15-fold between days 22 and 36 of gestation (Norman & Poyser, 1998b).

Rice *et al.* (1995) have proposed that increased PG synthesis by the placenta during pregnancy, particularly the second half of pregnancy, may have a role in fetal maturation and may enhance the transport of essential substances such as glucose, oxygen and amino acids, to the fetus.

1.1.5 Parturition

Prostaglandins have been used in human pregnancy since 1967. They were first employed for the induction of labour (Karim *et al.*, 1968), and then later used for pregnancy termination (Karim & Filshie, 1970), for preparing the cervix for induction (Calder & Embrey, 1973) and for the treatment of post-partum haemorrhage (Tagaki *et* al., 1976; Keirse *et al.*, 1989).

It has been hypothesised from studies on the sheep that, when the fetus enters its rapid growth period (two thirds of the way through gestation), it puts an increased metabolic demand on the placenta which, in turn, stimulates the production of PGE_2. Increased PGE_2 output then stimulates the fetal pituitary-adrenal axis leading to parturition (Thorburn, 1991). Removal of the ovine fetal pituitary reduces PGE_2 output from the placenta (Thorburn, 1991), which is consistent with the view that, in the pregnant sheep, the fetal pituitary gland exerts direct control on placental steroid and PG synthesis. PGE_2 output from the placenta and from fetal membranes has been observed to increase in the final stages of gestation in the sheep (Risbridger *et al.*,

1985; Rice *et al.*, 1988), rabbit (Elliot *et al.*, 1984), pig (Rice *et al.*, 1989) and the guinea-pig (Schellenberg & Kirby, 1997). Addition of arachidonic acid does not increase PGE_2 output any further from the sheep placenta (Risbridger *et al.*, 1985) or the guinea-pig placenta (Schellenberg & Kirby, 1997), and it has been suggested that the cyclooxygenase activity of PGHS may be the rate-limiting step in PG production by the placenta.

It is not clear what regulates PG synthesis during the last trimester of gestation but the fetal pituitary hormones have been suggested, including the gonadotrophins (Thorburn, 1991). Fetal pituitary hormones, particularly gonadotrophins regulate steroid and PG synthesis by the placenta (Thorburn *et al.*, 1989). It has been proposed that luteinising hormone (LH) may induce the cyclooxygenase activity of the PGHS enzymes in the placenta as it does in the ovary (Thorburn, 1991). In sheep an increase in PGE_2 by the fetal trophoblast activates the fetal hypothalamic-pituitary-adrenal (HPA) axis leading to an increase in fetal cortisol. High cortisol leads to an increase in placental 17α-hydroxylase activity, which decreases maternal progesterone while increasing maternal oestrogen. An increase in the oestogen:progesterone ratio results in increased PGE_2 release from the placenta thus increasing uterine activity and parturition (Thorburn, 1991). Gonadotrophin releasing hormone (GnRH) may also have a role in PG synthesis by the placenta. Hanig *et al.* (1982) demonstrated that GnRH stimulated $PGF_{2\alpha}$ production by human placental homogenates. GnRH has also been observed to stimulate PG production by the human placenta (Kang *et al.*, 1991). It has been postulated that GnRH may be stimulating the production of a gonadotrophin within the guinea-pig placenta, and this gonadotrophin may be related to human chorionic gonadotrophin (Humphreys *et*

al., 1982). However, GnRH has also been observed to inhibit PG output from human placenta (Siler-Khodr *et al.*, 1986a, 1986b; Kang *et al.*, 1991). Further investigation is required to determine the role of GnRH in PG production by the placenta.

The type of placenta produced depend on the species and the guinea-pig placenta is the same as the human placenta in this respect i.e. it is discoidal and haemochorial in structure. Ovarian progesterone is necessary for maintenance of pregnancy and this is the role of the corpus luteum in early pregnancy in both human and guinea-pigs. Gestation in the guinea-pig last between 62-70 days. The corpora lutea of the guinea-pig are maintained until day 58-67, but are not essential for maintenance of pregnancy beyond day 25 (Bland & Donovan, 1969). Luteal-placental shift occurs on day 25 of pregnancy when the placenta takes over progesterone production. However, luteal progesterone production is functional until day 40 of pregnancy after which progesterone secretion gradually declines (Heap & Deansesly, 1966). The guinea-pig and the human are the only species where progesterone levels remain high as parturition approaches (see Horton & Poyser, 1976). It is believed that the guinea-pig placenta is a useful model to use for the studies carried out in this thesis.

1.2 PURINOCEPTORS

1.2.1 The History of Purinoceptors

The potent cardiovascular effects of adenosine led to an interest in the synthesis of new adenosine analogues (Fredholm *et al.*, 1994). Biochemical evidence for multiple receptor types for adenosine was provided by the demonstration that adenosine analogues increased cAMP production in some preparations, while decreasing it in others. Thus adenosine receptors were classified as A_1 and A_2 (van Calker *et al.*, 1979) or Ri and Ra (Londos *et al.*, 1980). More recently the A_2 receptor has been sub-divided into an A_{2A} and an A_{2B} receptor. Adenosine (A) receptors are G protein-linked receptors. A_2 receptors have been defined on their ability to stimulate adenylate cyclase, by being coupled to the G protein Gs. Response to A_1 receptor activation is blocked by pertussis toxin and this is compatible with Gi/Go involvement. A third adenosine receptor has been classified as A_3. The existence of four separate adenosine receptors is now well established, A_1, A_{2A}, A_{2B} and A_3 (Girdlestone & Watson, 1996).

ATP was also observed to have important cardiovascular effects (Green & Stoner, 1950), and Burnstock (1972) postulated the existence of specific ATP receptors. Receptors for adenosine, ATP and ADP are collectively known as purinoceptors. In 1978, Burnstock classified these as P1 (now known as A receptors) and P2 purinoceptors for adenosine and ATP, respectively.

The P2 receptors until recently were divided into the sub-types P_{2X}, P_{2Y}, P_{2U}, P_{2t}, P_{2Z} and P_{2d} based mainly on different agonist potencies. Initial work was carried out by Burnstock & Kennedy (1985) who discriminated between the two major types of

receptor, P_{2X} and P_{2Y}, by their response to ATP and different ATP analogues. P2 classification was thought to be unsatisfactory for the long term (Fredholm *et al.*, 1994), as it had been shown that the P_{2Y} receptor has different agonist pharmacology in different tissues (Burnstock 1991, Fischer *et al.*, 1993). Thus the 'classic' P_{2Y} purinoceptor required further sub-classification.

In 1991, Dubyak proposed that there were two different signal transduction mechanisms for P2 purinoceptors. This observation and the cloning of these receptors, which began with cloning of the P_{2Y} receptor from chick brain (Webb *et al.*, 1993), led to a more logical classification of receptor types and sub-types (see Fredholm *et al.*, 1997). Abbrachio & Burnstock (1994) reclassified the purinoceptors into 2 main families, P2Y and P2X. The international union of pharmacology committee on receptor nomenclature and drug classification (NC-IUPHAR) also classified purinoceptors into these 2 families. The P2Y family of receptors, which are G protein-linked, often couple to stimulate phospholipase C and inositol triphosphate (IP_3) formation (O'Conner *et al.*, 1991). This receptor family contains several sub-types ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$), most of which have been cloned in humans.

There is evidence that receptors exist that are structurally similar to receptors for ATP but strongly prefer UTP or UDP (Communi & Boeyneams, 1997; Harden *et al.*, 1997). These receptors have previously been classified as the P_{2U} or 'nucleotide' or 'pyramidine' receptors (O'Conner *et al.*, 1991; Dubyak, 1991). Recently it has been suggested that distinct pyrimidinoceptors do exist although they do not appear to constitute a separate receptor family of their own (Communi & Boeynaems, 1997). It has now been proposed that this family of receptors be named P2 receptors rather

than P2 purinoceptors. A P2 receptor is now described as a receptor for a purine or pyrimidine nucleotide (or dinucleotide). The $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are all stimulated by UTP.

The P2X family of receptors are ion channels permeable to Na^+, K^+ and Ca^{2+} (Bean, 1992), and has been sub-divided into 7 different receptor types. P_{2Z} is now considered to be a member of the P2X family ($P2X_7$) (see Fredholm, 1997). $P2X_7$ mediates the response to ATP in mast cells (Dahlqvist & Diamant, 1974) and macrophages (Steinberg & Silverstein, 1987).

Kennedy & Leff (1995) suggested that the classification of receptors based on agonist potencies could be wrong due to the breakdown of ATP and 2-methylthio-ATP by ecto-nucleotidases. Recent studies have shown that the potencies of ATP and 2 methylthio-ATP decrease between 100- to 1000-fold by breakdown and, when breakdown of these compounds is prevented, that both are more potent than αβ methylene-ATP at the P2X receptor. ATP and 2 methylthio-ATP are broken down by nucleotidases but αβ methylene-ATP is relatively resistant (Welford *et al.*, 1986; Welford *et al.*, 1987) and αβ methylene-ATPis generally considered to be less potent than ATP or 2 methylthio-ATP. At the moment there are limited, highly selective, metabolically stable agonists and antagonists available to classify purinoceptors.

In summary, in addition to their role in energy metabolism, purines, especially adenosine and adenine nucleotides, produce a wide range of pharmacological effects mediated by activation of cell surface receptors. Studying these receptors is complicated by the rapid metabolism and uptake of the agonists, thus making it difficult to determine which compound specifically is producing the effect.

1.2.2 G Proteins and Second Messengers

G proteins are guanine nucleotide binding proteins, which provide a link between the receptor site and intracellular activity. They are membrane-associated compounds and are heterotrimers consisting of α, β and γ sub-units. They respond to ligand-activated receptors by dissociating into their component α- and βγ-sub-units (Axelrod *et al.*, 1988). Therefore, G proteins exist in 2 states, namely 'active' with GTP bound to the α-sub-unit or 'inactive' with GDP bound to the β-sub-unit. The GTP bound α-sub-unit then activates a second messenger system which can stimulate a number of processes in the cell (Axelrod *et al.*, 1988). These second messenger systems include activation of regulatory proteins such as adenylate cyclase, cGMP phosphodiesterase and some voltage-sensitive ion channels. In some situations, the βγ-sub-unit has also been observed to activate enzymes (see Axelrod *et al.*, 1988).

There are a number of different G proteins, Gs, Gi, Gt and Go. G proteins can be identified by their reactions to specific toxins. Cholera toxin induces ADP ribosylation of Gs which under normal conditions stimulates adenylate cyclase. The toxin stabilises the dissociated form of the G protein i.e. the GTP bound α-sub-unit which leads to persistent activation of adenylate cyclase. Another toxin, pertussis toxin, causes ADP ribosylation of Gi proteins, which normally inhibit adenylate cyclase. Pertussis toxin prevents the coupling of the G protein with the activated receptor and stabilises the G protein in its associated state, thus preventing inhibition of adenylate cyclase.

There is evidence that PLA_2 and PLC are activated through G proteins (Axelrod *et al.*, 1988). Toxin effects on a particular effector system may be an indication of regulation by a GTP-binding protein (Axelrod *et al.*, 1988). It has been reported that PLA_2 is activated by a G protein that is inhibited by pertussis toxin. Pertussis toxin inhibits PG synthesis in a number of tissues, suggesting the involvement of G proteins in their synthesis (Burch *et al.*, 1986; Burgoyne *et al.*, 1987; Axelrod *et al.*, 1988; Wang *et al.*, 1988; Nakashima *et al.*, 1988). Receptor-mediated release of arachidonate has been demonstrated in fibroblasts, platelets and neutrophils (Samuelsson *et al.*, 1978; Hirata *et al.*, 1979; Ohta *et al.*, 1985; Bokoch *et al.*, 1984). However, in some of these experiments arachidonate release may not have been by direct action of PLA_2 but by a prior action of PLC (Bokoch *et al.*, 1984). Experiments with noradrenaline-stimulated thyroid cells and light activated rod outer segments (ROS) of bovine retina have provided evidence for the direct, G protein-dependent regulation of PLA_2 which does not involve PLC (see Axelrod *et al.*, 1988). It is probable that arachidonic acid release by PLC and PLA_2 involves the activation of different G proteins. Pregnancy suppresses G protein coupling to phosphoinositol hydrolysis in the guinea-pig myometrium, which may contribute to a decrease in contractile sensitivity during pregnancy (Arkinstall & Jones, 1990).

1.2.3 ATP, Prostaglandins and the Uterus

Adenosine and adenine nucleotides are potent stimulators of PG release in a number of critical organs (Needleman *et al.*, 1974) and have been shown to stimulate contraction and relaxation in various tissues. These agonists mediate their effects via A (adenosine) receptors and P2 purinoceptors. Both A and P2 receptors have been

identified in the guinea-pig uterus and their activation causes contraction of uterine smooth muscle (Smith *et al.*, 1988). Previously, it has been shown that the smooth muscle of the guinea-pig uterus responds by contracting to both ATP and adenosine (Moritoki *et al.*, 1979) and appears to be one of the few smooth muscle tissues that responds this way. ATP-induced contractions of the guinea-pig uterus are blocked by indomethacin (an inhibitor of PGHS), PG antagonists and ATP antagonists (Moritoki *et al.*, 1979).

Scheimann *et al.* (1991) reported that A_1 receptor activation stimulates PG synthesis in guinea-pig myometrium and observed increased 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ production when this receptor type was stimulated. ATP has also been observed to stimulate $PGF_{2\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 production by the rabbit myometrium (Suzuki, 1991). Pregnant rabbit myometrium appears to be more sensitive to adenosine and adenine nucleotides than non-pregnant myometrium, which could be due to an increase in the number of receptors or an increase in the affinity of the receptors (Suzuki, 1991).

There are conflicting views as to whether the contractile actions of adenosine and ATP on the uterus involve PGHS, the enzyme responsible for the conversion of arachidonic acid to PGH_2. Bradley *et al.* (1993) suggests that PGHS is not necessary to produce the contractile response of the guinea-pig uterus to adenosine, as indomethacin had no effect on adenosine-induced contractions of the guinea-pig myometrium. Moritoki *et al.* (1979) and Suzuki (1991) concluded otherwise and demonstrated that indomethacin inhibited adenosine- and ATP-induced contractions of the guinea-pig uterus, respectively. This difference in opinion may be due to

different experimental techniques, and whether PGHS is involved remains to be resolved.

ATP and adenosine could be linked to stimulation of phosphoinositide hydrolysis (PI), leading to inositol triphosphate (IP_3) and diacylglycerol (DAG) production and the subsequent release of PGs. Activation of adenosine receptors in uterine tissue results in increased phosphoinositide metabolism (Scheimann *et al.*, 1991) and increased intracellular calcium concentrations (Bradley & Buxton, 1991). Suzuki (1991) demonstrated that ATP stimulates diacylglycerol formation in myometrial strips, which is indicative of PLC activation. Inositol 1,4,5-triphosphate (IP_3) is a breakdown product of PLC-mediated phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis. IP_3 releases calcium from the sarcoplasmic reticulum of muscle cells (Schrey *et al.*, 1988), and is involved in smooth muscle contraction. IP_3 has been shown to facilitate uterine contractions in a dose-dependent manner by releasing calcium from pregnant rat (Kanmuru *et* al., 1988) and pregnant bovine (Carsten & Miller, 1985) sarcoplasmic reticulum microsomes. Formation of prostanoids could result from the liberation of arachidonic acid from DAG, another product of phosphoinositide hydrolysis, if the receptor is coupled directly to PLC activation (Scheimann *et al.*, 1991). Addition of PLC has been shown to increase PGE_2 production by the guinea-pig uterus (Poyser, 1987a). P2Y receptors are linked to G proteins and PLC in a number of tissues including erythrocytes, aortic endothelial cells and the lungs (Berrie *et al.*, 1988; Parr *et al.*, 1994; Lazarowski & Harden, 1994; Wilkinson *et al.*, 1994).

In the experiments of Scheimann *et al.* (1991), increased production of inositol phosphates was not blocked by pertussis toxin (PTx), indicating adenosine (A) receptor coupling to a PTx-insensitive G protein. This is consistent with previous work implicating the role of a PTx-insensitive G proteins in regulating receptor activated increases in inositol phosphates in muscle tissue (Masters *et al.*, 1985; Marc *et al.*, 1988) and suggesting a role for calcium release in the contractile action of adenosine (Scheimann *et al.*, 1991). Overall, the ability of ATP to exert its effects via G protein indicates the presence of A receptors and/or P2Y purinoceptors, which are coupled to G proteins (O'Conner *et al.*, 1991). P2X receptors cannot be involved since these are ligand-gated ion channels (Bean, 1992). One of the main aims of this thesis has been to study the effects of ATP, ATP analogues and adenosine on prostaglandin production by the non-pregnant guinea-pig uterus, particularly by the endometrium. This is because it has been reported that ATP-induced contractions of the guinea-pig uterus are inhibited by indomethacin and by removal of the endometrium (Piper & Hollingsworth, 1996)

1.3 PROSTAGLANDIN H SYNTHASE (PGHS)

1.3.1 Isoforms of Prostaglandin H Synthase (PGHS)

Prostaglandin endoperoxide synthase (PGHS) is a membrane-bound, glycoprotein complex and is the enzyme required to produce PGs. PGs of the 2-series are produced from arachidonic acid, and the first step is the conversion of arachidonic acid to PGH_2 by PGHS (see Fig. 1.1.2.2). This enzyme has two actions. Initially, arachidonic acid is converted to the unstable PGG_2 by the cyclooxygenase activity of PGHS, and then the peroxidase activity converts PGG_2 to PGH_2. PGH_2 is the key intermediate in the biosynthesis of the prostaglandins and the final form of prostaglandin produced is dependent on the cell type and the enzymes they contain (Samuelsson *et al.*, 1978).

Recently two isoforms of this enzyme have been identified, cloned and sequenced (DeWitt & Smith, 1988; Merlie *et al.*, 1988; Kujubu *et al.*, 1991; Wong & Richards, 1991; O'Banion *et al.*, 1992). They have been named PGHS-1 and PGHS-2. The two enzymes catalyse identical reactions, and have similar amino acid sequences, catalytic properties and structures. PGHS-1 is expressed constitutively in prostanoid producing cells. PGHS-1 mRNA and protein are found in most tissues under normal conditions. PGHS-2 is the inducible form of this enzyme, and its mRNA and protein levels are very low or even undetectable in most tissues under normal circumstances (Feng *et al.*, 1993; Kargman *et al.*, 1994). PGHS-2 is expressed when cells are exposed to mitogens such as growth factors or cytokines. These differences suggest different physiological roles for these isozymes in the body. It has been proposed that PGHS-1 is responsible for cellular housekeeping such as vascular homeostasis, water

resorption in the collecting tubule of the kidney and control of gastric acid secretion in the stomach (see Otto & Smith, 1995). PGHS-1 is continually available to these tissues for rapid synthesis of PGH_2. PGHS-2 is thought to be involved in differentiative responses including inflammatory processes, ovulation and mitogenesis. PGHS-1 and PGHS-2 are found on the endoplasmic reticulum and on the nuclear envelope (Regier *et al.*, 1993) but it has been proposed that PGHS-1 is predominantly located on the endoplasmic reticulum, while PGHS-2 is localised predominantly on the nuclear envelope (see Otto & Smith, 1995).

The question is raised as to why there are two forms of the same enzyme that both carry out the same reaction? A possible explanation comes from experiments in fibroblasts and macrophages (Reddy & Herschman, 1994). Reddy & Herschman, (1994) observed that these cells can constitutively produce PGHS-1 and be induced by TPA (a protein kinase C activator) to produce PGHS-2. When expression of PGHS-2 protein in response to TPA was blocked, no PGs were produced even though arachidonic acid mobilisation was observed. It was hypothesised that PGHS-1 can utilise exogenous arachidonic acid but not that released due to TPA. Therefore, the arachidonic acid released by TPA must be specifically channelled to PGHS-2 (Reddy & Herschman, 1994). Therefore, different pools of arachidonic acid may exist in tissues and these pools are specifically channelled to different PGHS enzymes (Reddy & Herschman, 1994). It has also been proposed that PGHS-1 and PGHS-2 could be linked to distinct prostaglandin synthases (Naraba *et al.*, 1998). Several lines of evidence have reported that the two isoforms of PGHS regulate different phases of prostanoid biosynthesis rather than exert overlapping functions in activated cells (Langenbach *et al.*, 1995; Murakami *et al.*, 1994; Reddy &

Herschman, 1994). PGHS-1, but not PGHS-2, functions in thromboxane generation by activated platelets (Langenbach *et al.*, 1995), and in immediate PGD_2 generation by IgE/Ag-activated mast cells (Murakami *et al.*, 1994; Reddy & Herschman, 1994), while PGHS-2 is an absolute requirement for delayed prostanoid generation, extending over several hours, elicited by pro-inflammatory stimuli, even though PGHS-1 co-exists in the same cell (Murakami *et al.*, 1994; Reddy & Herschman, 1994).

Expression of PGHS has been proposed as the rate-limiting step in PG synthesis (Poyser, 1973). However, others disagree because an immediate rise in PG synthesis is seen when exogenous arachidonic acid is added, for example, to intrauterine tissues (Risbridger *et al.*, 1985; Bennett *et al.*, 1992; Schellenberg & Kirby, 1997). However, this was not the case in all intrauterine tissues studied. PG output from the placenta does not increase in the presence of exogenous arachidonic acid (Risbridger *et al.*, 1985; Schellenberg & Kirby, 1997).

PGHS exhibits self-catalysed breakdown. As PGs are formed, the enzyme catalysing the reaction is inactivated (Lands, 1979). Therefore, this suggests that fresh PGHS enzyme must be synthesised in order to produce PGs. It appears that protein synthesis may be necessary for prostaglandin production. The message and the protein for PGHS-1 are found in most cells at stable levels, while the message and the protein for PGHS-2 are low or undetectable but are strongly and rapidly induced in response to cytokines and mitogens (see Kulmacz, 1998). Important regulation of PGs occur at the level of catalytic activity with different control of PGHS-1 and PGHS-2. PGHS-2 is not required to overcome insufficient catalytic capacity but supplements the latent enzyme, PGHS-1, with a catalytically active enzyme, PGHS-2

(see Kulmacz, 1998). Once synthesised by the action of PGHS enzymes, prostaglandins act at specific 7 transmembrane domain prostaglandin receptors. Pharmacologically, the prostanoid receptors were initially categorised according to their endogenous ligands. DP receptors are activated by PGD_2, EP receptors by PGE_2, FP receptors by $PGF_{2\alpha}$, IP receptors by PGI_2 and TP receptors by TXA_2. The EP receptors were further subdivided into four distinct receptor subtypes, $EP_{1\text{-}4}$ (see Pierce & Regan, 1998). All prostanoid receptors are G protein coupled receptors. EP_3 receptors are coupled to inhibition of adenylyl cyclase, while DP, IP EP_2 and EP_4 recpetors are coupled to the activation of adenylyl cyclase. The EP_1, FP and TP receptors are coupled to phosphatidylinositol hydrolysis. Recently different isoforms of the EP_1, EP_3, FP and TP receptors (see Pierce & Regan, 1998). Therefore, there are several possible signal transduction mechanisms that may be involved in the actions of PGs.

1.3.2 PGHS and the Placenta

Prostaglandin synthesis is regulated *in vivo* by the availability of substrate i.e. arachidonic acid, and by the level of catalytically active PGHS (Smith *et al.*, 1991; Challis & Olson, 1988; Shimokawa & Smith, 1991). The change in expression of PGHS is a rate-limiting step in PG production (Van den Ouderaa *et al.*, 1979). As mentioned previously, two isoforms of the PGHS enzyme (PGHS-1 and PGHS-2) have been identified, cloned and sequenced (DeWitt & Smith, 1988; Merlie *et al.*, 1988; Kujubu *et al.*, 1991; O'Banion *et al.*, 1992). It has previously been hypothesised that increased PG synthesis by intra-uterine tissues is a result of increased tissue content of PGHS (Rice *et al.*, 1990). This could involve an increase

in PGHS synthesis, increased half-life of PGHS or increased affinity of PGHS for its substrate (Rice *et al.*, (1992).

Previous workers have attributed increased PG production by placenta to increased PGHS activity which, in turn, is dependent on the gestational age (Rice *et al.*, 1988; Risbridger *et al.*, 1985). Poyser (1993) showed that guinea-pig placental homogenates have a high capacity for synthesising PGs. This reflects a high concentration of PGHS. However, more recent work has concentrated on which isoform of the PGHS enzyme is responsible for increased prostanoid synthesis from the placenta throughout gestation. Gibb *et al.* (1996) observed an increase in PGHS-2 mRNA throughout gestation in ovine placenta, particularly by the trophoblast, while no change was seen in PGHS-1 mRNA. This is consistent with the results of other workers (Freed *et al.*, 1995; Rice *et al.*, 1995). It appears likely that the increase in PGHS-2 mRNA observed was responsible for the increased PGHS-2 immunoreactivity reported by Wimsatt *et al.* (1993) and the increased PGHS activity seen during pregnancy (Rice *et al.*, 1988). It has been proposed that increased PGHS activity during pregnancy may be associated with an increase in phospholipase activity (Bennett *et al.*, 1992; Rice *et al.*, 1994). One of the aims of this thesis is to establish which isoform of PGHS is responsible for increased PG output from the guinea-pig placenta after day 25 of pregnancy.

1.4 THE INVOLVEMENT OF PHOSPHOLIPASE ENZYMES, CALCIUM, CALMODULIN AND PROTEIN KINASE C IN PROSTAGLANDIN PRODUCTION BY THE PLACENTA.

1.4.1 Phospholipase Enzymes and Calcium

It is generally considered that the release of bound arachidonic acid from membrane phospholipids is the rate-limiting step in the synthesis of prostaglandins (Vogt, 1978), although changes in the concentration of PGHS are able to regulate the amount of PG synthesised from free arachidonic acid (Herschman, 1996). Release of arachidonic acid from phospholipids is brought about by the action of phospholipase (PL) enzymes. At present there are four major groups of phospholipases, PLA_1, PLA_2, PLC and PLD (Dennis, 1983). Arachidonic acid can be released from phospholipids indirectly from inositol phospholipids by the sequential actions of phospholipase C (PLC), diglyceride lipase and monoglyceride lipase.

PLC is a phosphodiesterase which can hydrolyse phosphatidylinositol-4,5-biphosphate (PIP_2), producing diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 causes mobilisation of intracellular calcium from the endoplasmic reticulum. DAG has a number of actions. It activates protein kinase C (PKC), which has been observed to stimulate the release of arachidonic acid from phospholipids and PG synthesis (Wijkander & Sundler, 1989; Zaker & Olson, 1989). DAG may also regulate arachidonic acid mobilisation through inhibition of one of the enzymes responsible for arachidonic acid uptake e.g. acyl transferase, thus increasing free available arachidonate (Fuse *et al.*, 1989). DAG can also be converted to

phosphatidic acid (PA) (Takuwa *et al.*, 1986). PLA_2 can release arachidonic acid from PA.

Arachidonic acid is predominantly found esterified to the *sn*-2 position of membrane phospholipids and PLA_2 is most likely responsible for its release and subsequent eicosanoid biosynthesis (Flower & Blackwell, 1976). PLA_2 mobilises arachidonic acid from phospholipids in a one-step reaction (Mayer & Marshall, 1993; Kramer *et al.*, 1993; Dennis, 1994; Mukherjee *et al.*, 1994). 'PLA_2' is rapidly becoming a large superfamily of distinct enzymes, and their products are important for signal transduction processes, eicosanoid and platelet activating factor (PAF) formation, membrane remodelling and general lipid metabolism. Two major groups of PLA_2 enzymes have been identified, extracellular or secretory PLA_2 (14-18 kDa) and intracellular or cytosolic PLA_2 (31-110 kDa) (Loeb & Gross, 1986; Davidson & Dennis, 1990). Secretory PLA_2 was discovered by Loeb & Gross (1986) and a second type of PLA_2 ($cPLA_2$) was discovered and observed to have a high molecular mass, which was greater than the molecular masses of the recognised $sPLA_2$ (Davidson & Dennis, 1990).

Secreted PLA_2 ($sPLA_2$) enzymes were originally sub-divided into three groups, I, II and III but only types I and II have been identified in mammalian tissues (see Dennis, 1997). A large number of new distinct PLA_2 enzymes have been recently discovered, and from those that have been sequenced, it is clear that they do not fit into the traditional groups. Two more mammalian forms of $sPLA_2$ have been identified. Type IIC PLA_2 has been identified in the rat and mouse testes (Chen *et al.*, 1994a) and type V PLA_2 has been identified in human, rat and murine macrophages (Chen *et al.*, 1994b; Chen *et al.*, 1994c; Balboa *et al.*, 1996). The major groups of PLA_2 have been

extended up to group IX, and include secreted and cytosolic PLA_2 enzymes that are both dependent and independent of calcium. Recently identified, but only partially characterised, was a 42 kDa PLA_2 from the human placenta (Buhl *et al.*, 1995).

Initially there was some controversy as to whether PLA_2 enzymes were calcium requiring enzymes (Withnall & Brown, 1982; Downing & Poyser, 1983; Moskowitz *et al.*, 1985). However, recent studies have observed that $sPLA_2$ enzymes can be activated by millimolar concentrations of calcium and do not demonstrate any selectivity for the fatty acids present in the sn-2 position of phospholipid substrate (Glaser *et al.*, 1993). $cPLA_2$ preferentially hydrolyses arachidonoyl phospholipids and can be activated by sub-micromolar concentrations of calcium ions and by phosphorylation by mitogen-activated protein kinase (MAPK) (Leslie, 1997; Clark *et al.*, 1991; Sharp *et al.*, 1991; Lin *et al.*, 1993; Kramer *et al.*, 1996). In mammalian cells both $sPLA_2$ and $cPLA_2$ have been reported to mediate liberation of arachidonic acid from tissue lipids in response to chemical signals (Lin *et al.*, 1992; Fonteh *et al.*, 1994).

$cPLA_2$ has no homology with known forms of $sPLA_2$ (Clark *et al.*, 1991; Sharp *et al.*, 1991) and is subject to diverse mechanisms of regulation. $cPLA_2$ can be quickly activated by post-translational mechanisms to rapidly mobilise arachidonic acid for PG production, but is also subject to increased synthesis and long term activation, leading to prolonged PG production (Leslie, 1997). Calcium and mitogen-activated protein kinase (MAPK) are capable of stimulating $cPLA_2$ activity but it has been demonstrated that full activation of $cPLA_2$ is only achieved when MAPK-induced phosphorylation is followed by increased calcium ion concentrations and translocation of the enzyme to the membrane (Leslie, 1997). $cPLA_2$ contains a

specific calcium-dependent phospholipid binding domain (CaLB) which shares homology with the C2 domain of PKC (Clark *et al.*, 1991; Sharp *et al.*, 1991).

Srinivasa *et al.* (1997) and Balsinde *et al.* (1996) suggested recently that functional $cPLA_2$ may be essential for $sPLA_2$ to function. $cPLA_2$ may modify the structure of the plasma membrane rendering it susceptible to $sPLA_2$. Type V $sPLA_2$ has been shown to stimulate PG production but prior activation with $cPLA_2$ is necessary (Balboa *et al.*, 1996; Balsinde *et al.*, 1996). Inflammatory stimuli often induce expression of type IIA $sPLA_2$ (Pfeilschifter *et al.*, 1993; Nakano *et al.*, 1990; Nakazato *et al.*, 1991) and $cPLA_2$ (Lin *et al.*, 1992) while anti-inflammatory agents down-regulate their expression and function (Nakano *et al.*, 1990; Lin *et al.*, 1992; Muhl *et al.*, 1992).

1.4.2 Phospholipases and the Placenta

As just described, multiple forms of PLA_2 have been identified and two functionally distinct groups characterised i.e. $sPLA_2$ and $cPLA_2$. To date only four types of PLA_2 have been identified in human intra-uterine tissues and the human placenta. These are $cPLA_2$, type II $sPLA_2$, type IV $sPLA_2$ and type V $sPLA_2$ (Seilhamer *et al.*, 1989; Bennett *et al.*, 1993; Freed *et al.*, 1997; Rice *et al.*, 1998). Recently Buhl *et al.* (1995) discovered a novel PLA_2 in the human placenta at term. It has a molecular mass of 42 kDa, which distinguishes it from $sPLA_2$ and $cPLA_2$ although it shares some properties with both enzymes. This PLA_2 enzyme prefers phosphatidylcholine (PC) as its substrate (Buhl *et al.*, 1991). This 42 kDa enzyme has been named type V $sPLA_2$ and contains a calcium-dependent lipid-binding domain. While $cPLA_2$ requires μM concentrations of calcium for activation, it can be further stimulated by

mM concentrations of calcium (Clark *et al.*, 1990; Leslie *et al.*, 1988; Kim *et al.*, 1991). Placental PLA_2 identified by Buhl *et al.* (1995) can be stimulated by 10 μM calcium but cannot be further stimulated by increased calcium concentrations. The cellular origin of this 42 kDa PLA_2 is not clear; it may be exclusively expressed in placental cells or may originate in some other maternal organ e.g. the uterus.

Expression of type II PLA_2 in the placenta has been studied in great detail. Type II PLA_2 mRNA, immunoreactive content and enzymatic activity have been identified and localised in several gestational tissues including the amnion, choriodecidua and placenta (Aitken *et al.*, 1990, 1992, 1996; Farrugia *et al.*, 1993; Andersen *et al.*, 1994). Type II PLA_2 is a major component of the net PLA_2 in the human placenta (Rice, 1995). It has been proposed that increased expression of type II PLA_2 may contribute to a labour-associated increase in eicosanoid formation by gestational tissues (Keirse *et al.*, 1977; Sellers *et al.*, 1981) and to increased type II $sPLA_2$ concentrations in maternal plasma (Rice *et al.*, 1992). Rice (1995) proposed that type II PLA_2 is released by human gestational tissues and acts extracellularly to promote phospholipid metabolism. A 2-fold increase in immunoreactive type II PLA_2 has been demonstrated in explants of the human term placenta (Farrugia *et al.*, 1997). Type II PLA_2 released from the placenta may enhance placental and/or vascular glycerophospholipid metabolism and eicosanoid formation (Farrugia *et al.*, 1997). Although other forms of PLA_2 enzymes have been observed in the placenta, particularly the human placenta, type II PLA_2 appears to be the dominant isoform (Freed *et al.*, 1997). Rice *et al.* (1994) reported that type II PLA_2 accounted for 60-80% of the total PLA_2 enzymatic activity in the human term placenta. A recent study by Rice *et al.* (1998) observed that extraction of type II PLA_2 from the human

placenta using antibodies was associated with an 82% decrease in PLA_2 enzymatic activity.

Expression of $cPLA_2$ mRNA has also been demonstrated in the human placenta but $cPLA_2$ mRNA expression was very low compared to the expression of type II PLA_2 mRNA. $cPLA_2$ mRNA expression was much higher in the human amnion (Freed *et al.*, 1997). However, the activity of $cPLA_2$ in the placenta may have an important role to play in the development of the fetus, as it has been demonstrated that $cPLA_2$ deficient mice have impaired female reproductive functions (Uozumi *et al.*, 1997).

Recent reports have described the molecular cloning and characterisation of a receptor for $sPLA_2$ in various mammalian species (Higashino *et al.*, 1994; Ishizaki *et al.*, 1994; Lambeau *et al.*, 1994; Ancian *et al.*, 1995). PLA_2 receptor mRNA has been identified in human gestational tissues and may contribute to the regulatory effects of $sPLA_2$ isozymes in these tissues (Moses *et al.*, 1998).

In cases of pre-eclampsia, there are reports of elevated PLA_2 enzymatic activity in homogenates of the placenta (see Aitken *et al.*, 1996) and elevated maternal plasma concentrations of immunoreactive type II PLA_2 (Lin *et al.*, 1995). Immunoreactive type II PLA_2 is also elevated in the maternal plasma of women delivering preterm (Rice *et al.*, 1992). This may be associated with bacterial infection.

Differential expression of PLA_2 enzymes may relate to different functional characteristics and different roles. For example, $sPLA_2$ enzymes act extracellularly and may affect phospholipid metabolism in the cell of origin, in adjacent cells or in distal cells. However, $cPLA_2$ functions only within the cell of origin (Freed *et al.*, 1997).

Norman & Poyser (1998a) demonstrated the presence of PLA_2 in the guinea-pig placenta and sub-placenta. However, the PLA_2 inhibitor, aristolochic acid, had very little inhibitory effect on PG production by the guinea-pig placenta and sub-placenta, while consistently inhibiting PG production by the guinea-pig endometrium. Several possible explanations for this include the fact that arachidonic acid may not be the rate-limiting step for PG synthesis by the guinea-pig placenta and sub-placenta. This has previously been suggested by Schellenberg & Kirby (1997) who observed no increase in PG output from the guinea-pig placenta in the presence of exogenous arachidonic acid. Alternatively, the release of arachidonic acid from the guinea-pig placenta may be dependent on a PLA_2 which is unaffected by aristolochic acid, or the release of arachidonic acid may be via a pathway that does not involve PLA_2 (Norman & Poyser, 1998b). Therefore, PLA_2 enzymes may contribute to cell membrane phospholipid metabolism and the formation of second messengers during pregnancy and at the time of labour and delivery (Rice, 1996). However, it remains to be seen which PLA_2 isozymes are active in the guinea-pig placenta.

1.4.3 Calmodulin and Protein Kinase C

Calmodulin is a 17 kDa cytosolic acidic protein which binds calcium at a ratio of 1:4. It functions in a variety of cells (Cheung, 1980) and, in some tissues, PLA_2 is a calmodulin-dependent enzyme (Wong & Cheung, 1979; Maskowitz *et al.*, 1983). Calmodulin may have a critical role in controlling PG production in many tissues. It has been suggested that calcium binds with calmodulin which in turn combines with PLA_2 and activates the enzyme to release arachidonic acid for PG synthesis from phospholipids (Poyser, 1985a). Calmodulin has previously been observed to play a

role in PG synthesis by the guinea-pig uterus (Poyser, 1985a, b; Riley & Poyser, 1987). The use of calmodulin antagonists, trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) significantly reduced PG output from the superfused guinea-pig uterus and the guinea-pig endometrium in culture (Poyser, 1985a, b; Riley & Poyser, 1987). It is considered that calcium binding to calmodulin creates a 'hydrophobic domain' on the surface of calmodulin, which is essential for the binding of the complex to its acceptor protein. This domain is assumed to be the binding site of TFP (Andersson *et al.*, 1983). Calmodulin is difficult to antagonise if it is already tightly bound to its acceptor protein i.e. PLA_2 (Spedding, 1983).

The transmission of extracellular signals into their intracellular targets is mediated by a network of interacting proteins that regulate a large number of cellular processes. It is well documented that phorbol esters stimulate the activation of protein kinase C (PKC) in a number of tissues. PKC has long been implicated in activation of arachidonic acid release, and hence PLA_2 activation. The PKC activator, phorbol 12-myristate 13-acetate (TPA), has no effect on the outputs of PGs from cultured day 7 and 15 guinea-pig endometrium (Riley & Poyser, 1987b) or from guinea-pig uterine horns superfused *in vitro* (Poyser, 1987a). However, PKC has been observed to increase arachidonic acid release in, and $PGF_{2\alpha}$ and PGE_2 production by human decidual cells (Schrey & Read, 1986). Phorbol esters also stimulate PGHS-2 mRNA expression in cultured human amnion cells (Zakar *et al.*, 1996). These differences could be due to species variation. PKC has been identified in human fetal membranes (Okazaki *et al.*, 1984) and in the rat uterus (Baraban *et al.*, 1985).

Mitogen-activated protein kinase (MAPK) is a protein involved in signalling mechanisms. During stimulus-initiated arachidonic acid release accompanied by an increase in the free intracellular calcium concentration, $cPLA_2$ is phosphorylated and activated by MAPK (Lin *et al.*, 1993). The $cPLA_2$ is then translocated from the cytosol to the perinuclear and endoplasmic reticulum membranes (Schievella *et al.*, 1995). Phosphorylation of $cPLA_2$ increases its activity but does not cause its translocation to the nuclear envelope. Ca^{2+} is required for this process. PKC increases calcium channel activity (Shearman *et al.*, 1989) and thereby activates PLA_2 in some cell types (Xing & Insel, 1996; Felder, 1995). Translocation of $cPLA_2$ to phospholipid membranes is crucial for its function because of the localisation of its substrate. Phospholipid membranes are also the location of several enzymes which are important for PG production e.g. PGHS (Morita *et al.*, 1995). An increased intracellular calcium concentration seems to be the crucial step for $cPLA_2$ translocation because many of the agents stimulate its activity by increasing the influx of extracellular calcium (Kramer *et al.*, 1995). Kan *et al.* (1996) observed an influx of calcium was necessary for translocation of $cPLA_2$ but not for phosphorylation of $cPLA_2$. Therefore, the second main aim of this thesis is to investigate some of the intracellular processes controlling placental PG production, using the guinea-pig placenta as a model.

It is evident that PGs are produced by the non-pregnant uterus and by the placenta during gestation. PG production by the uterus has been implicated in a number of physiological processes including luteolysis and ovulation. However, little is known about the mechanisms involved in their production. Piper & Hollingsworth (1996) have suggested that ATP and ATP analogues stimulate PG synthesis from the guinea-

pig uterus. ATP may be stimulating PG production via P2 purinoceptors. This has been investigated using the non-pregnant guinea-pig uterus as a model.

PGs may also have a role in placental function, the control of placental and uterine blood flow, and in parturition (Hoedemaker *et al.*, 1991). Pre-eclampsia of pregnancy is associated with reduced PGI_2 production by the placenta (Walsh *et al.*, 1985). Therefore, a number of factors that may be involved in regulating PG production by the guinea-pig placenta have been investigated in this thesis. These include a study to determine whether PGHS-1 and/or PGHS-2 are important for PG production by the guinea-pig placenta and whether protein synthesis is necessary for this PG Production. The involvement of intracellular and extracellular calcium has been investigated. Calcium may be necessary for the activation of phospholipase A_2 (PLA_2). Calmodulin may also be a requirement for PLA_2 activation and PG synthesis. Therefore, the effects of two calmodulin antagonists on PG production by the guinea-pig placenta were studied. Protein kinase C (PKC) has long been implicated in arachidonic acid release, and hence PLA_2 activation. The effect of a PKC activator on PG output from the guinea-pig placenta was investigated. The role of gonadotrophin releasing hormone (GnRH) in PG production by the guinea-pig placenta was studied also.

These studies may help to clarify the mechanisms involved in PG synthesis from the guinea-pig uterus and placenta.

1.5 SPECIFIC AIMS

In summary, the specific aims of the studies performed in this thesis are:

1. To investigate the effects of adenosine, ATP and ATP analogues on PG production by the guinea-pig uterus, particularly the endometrium.

2. To investigate whether prostaglandin H synthase-1 or -2 is the predominant form of the enzyme responsible for PG output from the day 22 and 29 guinea-pig placenta and sub-placenta.

3. To determine whether protein synthesis is necessary for PG synthesis by the day 29 guinea-pig placenta and sub-placenta.

4. To investigate the calcium requirement for PG production by the day 29 guinea-pig placenta and sub-placenta.

5.To investigate the roles of calmodulin and protein kinase C in PG production by the day 29 guinea-pig placenta and sub-placenta.

6. To investigate a possible role for Gonadotrophin releasing hormone (GnRH) in PG production by the day 29 guinea-pig placenta and sub-placenta.

These studies should provide further information concerning the control of prostaglandin production by the uterus and placenta.

SECTION TWO

2. METHODS & MATERIALS

INTRODUCTION

Many of the general methods used in this project are common to a number of experiments including uterine superfusion, prostaglandin extraction, tissue culture and homogenisation of guinea-pig uterus and placenta. These methods are described here in detail.

2.1 GENERAL METHODS

2.1.1 GUINEA-PIG UTERINE SUPERFUSION

Female Dunkin-Hartley guinea-pigs (600-900 g) were examined daily and a vaginal smear was taken when the vagina was open. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification is at a maximum. All animals exhibited at least three normal cycles (about 16-18 days long) before being used. Guinea-pigs were killed by stunning and incising the neck. Experiments were carried out on day 7 of the oestrous cycle. The uterus was removed and separated into its two uterine horns. Each uterine horn was blotted dry, weighed and then cut longitudinally along the mesometrial side. These were then suspended in separate organ baths with one end attached to an isotonic lever under 2 g tension. Each horn was superfused independently with Krebs' solution (see Section 2.2.7f for composition) at 5 ml/min at 37°C, as described by Poyser & Brydon

(1983). After equilibrating for 60 min, superfusate samples were collected every 10 min over the next 80 min (8 samples per uterine horn) or 100 min (10 samples per uterine horn). The test uterine horn was superfused with the agonist between 90-110 min (experiments consisting of 8 samples), or with the antagonist between 90-110 min followed by the agonist plus antagonist between 110-130 min (experiments consisting of 10 samples). The control uterine horn was superfused with Krebs' solution only throughout the 140 or 160 min superfusion period. After collection, the pH of each sample was lowered to 4.0 using 1 M HCl and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate, according to the method described by Poyser (1972). The two extracts were combined (total volume 100 ml) and the samples were evaporated to dryness at 50°C in a rotary evaporator and re-dissolved in 10 ml of ethyl acetate. The recoveries of $PGF_{2\alpha}$ and PGE_2 by this method are >90%, and the recovery of 6-keto-$PGF_{1\alpha}$ is >80% (Poyser & Scott, 1980; Swan & Poyser, 1983). Samples were stored at -20°C until their PG content was measured.

2.1.2 GUINEA-PIG ENDOMETRIAL AND MYOMETRIAL CULTURE

Culture experiments using guinea-pig uterus were carried out according to the method of Baker & Neal (1969) and modified for the culture of endometrial tissue by Abel & Baird (1980) and Ning *et al.* (1983). Guinea-pigs were killed on day 7 of the oestrous cycle by stunning followed by exanguination. The uterine horns were removed under aseptic conditions and placed in sterile culture dishes containing tissue culture medium (TCM). TCM was made prior to use and consisted of 500 ml modified Medium M199 plus Earles salts (Table 2.1.2.1 for composition), L-

Table 2.1.2.1 Composition of Medium 199 modified with Earle's salts and 2.20 g/l sodium bicarbonate without glutamine. Storage Temperature 2-8 °C

Ingredient	**mg/litre**	**Ingredient**	**mg/litre**
L-Alanine	25.00	l-Inositol	0.05
L-Arginine.HCl	70.00	Menadione, $NaHSO_3.3H_2O$	0.019
L-Aspartic acid	30.00	Nicotinamide	0.025
L-Cysteine.HCl.H_2O	0.11	p-Aminobenzoic acid	0.05
L-Cystine.2HCl	26.00	Pyridoxine.HCl	0.05
L-Glutamic acid	75.00	Riboflavin	0.01
L-Glutamine	100.00	Thiamine.HCl	0.01
Glutathione	0.05	DL-αTocopherolphosphate disodium salt	0.01
Glycine	50.00	$CaCl_2$ (anhydrous)	200.00
L-Histidine.HCl.H_2O	21.88	$Fe(NO_3)_3.9H_2O$	0.72
L-Hydroxyproline	10.00	KCl	400.00
L-Isoleucine	20.00	$MgSO_4$ (anhydrous)	97.7
L-Leucine	60.00	Vitamin A acetate	0.14
L-Lysine.HCl	70.00	$NaHCO_3$	2200.00
L-Methionine	15.00	$NaH_2PO_4.H_2O$	140.00
L-Phenylalanine	25.00	Adenine sulphate	10.00
L-Proline	40.00	Adenylic acid	0.20
L-Serine	25.00	ATP, disodium salt	1.00
L-Threonine	30.00	Cholesterol	0.20
L-Tryptophan	10.00	2-Deoxyribose	0.50
L-Tyrosine disodium salt.$2H_2O$	57.66	D-Glucose	1000.00
L-Valine	25.00	Guanine.HCl	0.30
Ascorbic acid	0.05	Hypoxanthine, Na	0.354
Biotin	0.01	D-Ribose	0.50
Calciferol	0.10	Sodium acetate (anhydrous)	50.00
D-Calcium pantothenate	0.01	Phenol red sodium salt	10.00
Chlorine chloride	0.50	Thymine	0.30
Folic acid	0.01	Tween 80	5.00
NaCl	6800.00	Uracil	0.30
Xanthine, Na	0.34		

glutamine (4 ml; 200 mM solution), amphotericin B (3 ml; 250 μg/ml solution) and kanamycin (3 ml; 10 mg/ml solution). Each horn was cut longitudinally and 'opened'. The endometrium was carefully cut away from the myometrium with a pair of fine dissecting scissors into small pieces approximately 1 mm^3. The myometrium was also cut into pieces approximately the same size. The endometrium and myometrium were placed on sterile lens tissue on top of sterile stainless steel gauze platforms in Petri dishes containing 4 ml TCM plus the appropriate agonist and/or the antagonist. The presence of the gauze platform and lens tissue allowed the tissue to receive the TCM through the capillary action of the lens paper without actually sitting in the TCM. Endometrium and myometrium were cultured separately and the amount of tissue in each dish ranged from 10-40 mg. Petri dishes were placed in racks (maximum of 8 dishes per rack). A control Petri dish containing the tissue without the agonist/antagonist was placed at the top and bottom of each rack. Racks were placed in modified Kilner jars and incubated for 24 h (Fig. 2.1.2.1). Each Kilner jar was pressurised to 0.7 kg cm^{-2} (10 lb in^{-2}) with 95% O_2: 5% CO_2. The small amounts of CO_2 present allowed a CO_2/HCO_3^- buffer system to be set up which helped maintain the pH of the medium at 7.4. The TCM was removed from the dishes after 2, 8 and 24 h, and was replaced with fresh medium plus agonist and/or antagonist after 2 and 8 h; the dishes were returned to the Kilner jars and re-gassed. After 24 h all tissue was blotted dry and weighed. Samples of TCM were stored at -20°C before being assayed for PGs without extraction.

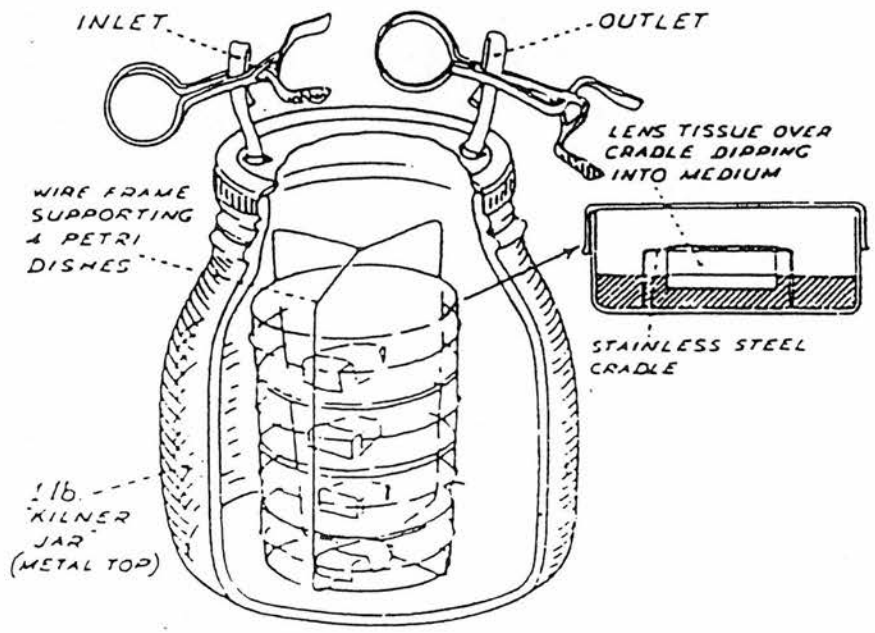


Figure 2.1.2.1 The arrangement for culturing uterine and placental tissue in a petri dish, and how the petri dishes were stacked and gassed (via the inlet and outlet tubes) in a Kilner jar.

2.1.3 HOMOGENISATION OF GUINEA-PIG ENDOMETRIUM AND MYOMETRIUM

These experiments were carried out using the endometrium and myometrium of the guinea-pig uterus after 24 h culture with agonist. The tissue was blotted dry, weighed and then homogenised using a Fisons glass homogeniser with 5 ml Krebs solution. The homoginiser was washed with a further 5 ml Krebs' solution, which was then combined with the original 5 ml (total volume of 10 ml) in a 25 ml conical flask. Each sample was allowed to incubate for 60 min in a Grant water bath at 37°C and 230 oscillations/min. The pH of each sample was lowered to 4.0 using 1 M HCl. Prostaglandins were extracted as described in Section 2.1.1 and the extract was evaporated to dryness on a rotary evaporator. Samples were re-dissolved in 5 ml ethyl acetate and were stored at -20°C until assayed for PGs.

2.1.4 CULTURE OF GUINEA-PIG PLACENTA AND SUB-PLACENTA

Animals were used on day 22 or 29 of pregnancy and killed as described in Section 2.1.1. The uterine horns were removed under aseptic conditions and placed in sterile culture dishes containing TCM. Each uterine horn was cut open to reveal between 1 and 4 embryos. The placenta was cut away from each embryo with a pair of fine dissection scissors. The placenta was then easily separated manually into the chorio-allantoic placenta (henceforth called the placenta) and the sub-placenta. Each tissue was cut into small pieces approximately 1 mm^3 and cultured for 24 h as described in Section 2.1.2. Placenta and sub-placenta were cultured separately with or without the drug treatment appropriate for the experiment. After 24 h all tissue was blotted dry and weighed. Samples of culture medium were stored at -20°C until assayed for PGs.

2.1.5 HOMOGENISATION OF GUINEA-PIG PLACENTA AND SUB-PLACENTA

Guinea-pig placentae were used on day 22 or 29 of pregnancy. The placentae were removed and separated from the sub-placenta as described in Section 2.1.4. Each tissue was roughly divided into equal portions for each experiment, blotted dry and weighed. The placentae were homogenised in 10 ml Krebs' solution containing a specific treatment. Samples were incubated and PGs were extracted as described in Section 2.1.1; the extracted PGs were re-dissolved in 5 ml ethyl acetate and stored at -20°C before being assayed for their PG content.

2.1.6 RADIOIMMUNOASSAY (RIA) OF PROSTAGLANDINS

2.1.6.1 Assay Procedure

The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ present in all samples were measured by radioimmunoassays (RIA). RIA was first developed in 1959 and is based on the antigen-antibody equilibrium reaction. It depends on labelled and unlabeled antigen competing for antibody binding sites.

The amount of tritiated prostaglandin [^{3}H]PG tracer used in each assay was sufficient to obtain counts of 15000-20000 per tube when counted in a scintillation counter for 4 min ($PGF_{2\alpha}$), 8 min (6-keto-$PGF_{1\alpha}$) and 10 min (PGE_2). [^{3}H]PG solution for each assay was made up from a stock solution of 5 μCi/ml stored in methanol or acetonitrile:water (9:1) at -20°C. An aliquot was taken and the solvents were blown off in a stream of warm air (45°C); the residue was re-dissolved in the appropriate diluent (see Section 2.2.7b-d) to the appropriate concentration. This concentration

was sufficient to allow 50 µl of the [^{3}H]PG solution to give the required number of counts.

All antibodies used in these experiments were raised in this laboratory. Antiserum was diluted to a concentration which gave 60 % - 80 % binding of [^{3}H]PG in the absence of non-radioactive PG (i.e. zero standards). The required dilution of antiserum was made from a 1:100 stock solution stored at 4°C. The specific concentration required for each assay was made up in the appropriate diluent.

Standard PG solutions were made from 1 µg/ml stock solutions in methanol stored at -20°C. 200 µl of stock solution was evaporated in a warm stream of air (45°C) and re-dissolved in 20 ml of the appropriate diluent (see Section 2.2.7) to give a final concentration of 10 ng/ml. A series of dilutions (9 for PGE_2 and 6-keto-$PGF_{1\alpha}$ and 10 for $PGF_{2\alpha}$) were prepared from the 10 ng/ml solution. Standard solutions ranged from 0.005 - 2.56 ng/ml ($PGF_{2\alpha}$) or 0.02 - 5.12 ng/ml (PGE_2 and 6-Keto-$PGF_{1\alpha}$) and were stored at -20°C until used. Standards were dispensed out in triplicate (0.5 ml) into 3 ml plastic tubes (Table 2.1.6.1.1). Tubes 1-4 were non-specific binding standards to determine the [^{3}H]PG binding to sites other than the specific PG binding sites, which include the plastic tube, non-specific sites on the antiserum and on other compounds in the assay. Tubes 5-8 were counting standards and together give an average of the counts per tube. The non-specific binding was always less than 10 % of the total binding for each assay. The last 4 tubes in each assay contained 0.5 ml of the appropriate diluent in duplicate (zero standards) and 0.5 ml of the PG standard solution (0.16 ng/ml or 0.32 ng/ml) also in duplicate so that the inter-assay coefficient of variation could be calculated.

Table 2.1.6.1.1 The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ used to execute each radioimmunoassay standard curve.

Tube number	(ng PG/Tube) $PGF_{2\alpha}$	(ng PG/Tube) PGE_2 / 6-keto-$PGF_{1\alpha}$
1-4	5	5
5-8	-	-
9-11	0.0025	0.01
12-13	0.005	0.02
14-16	0.001	0.04
17-19	0.02	0.08
20-22	0.04	0.16
23-25	0.08	0.32
26-28	0.16	0.64
30-32	0.32	1.28
33-35	0.64	2.56
36-38	1.28	-

The stored samples were dispensed out in duplicate in the appropriate volume. If stored in ethyl acetate, this was blown off in a warm stream of air at 45°C. The appropriate diluent was then added (0.5 ml) so that the volume was the same in the standards and in the sample tubes. Samples stored in TCM were dispensed out without extraction and 100 µl of TCM was added to all standards and the zero standards to minimise the effect of the medium on the assay.

All tubes were then treated the same way throughout the assay. [^{3}H]PG solution (50 µl) was added to all tubes (except to the counting standards), followed by 50 µl of antibody. The tubes were then 'whirlimixed' and incubated at room temperature for 1 h ($PGF_{2\alpha}$) or 2 h (PGE_2 and 6-keto-$PGF_{1\alpha}$). After incubation, normal rabbit serum (NRS; 50 µl; 1:140 dilution for the 6-keto-$PGF_{1\alpha}$ assay and 1:100 dilutions for the $PGF_{2\alpha}$ and PGE_2 assays) and donkey anti-rabbit serum (DARS; 50 µl; 1:10 dilution for all PG assays) were added; the tubes were 'whirlimixed' again and incubated overnight at 4°C. NRS was added to ensure that, at dilutions of antisera used, there was sufficient gamma-globulin present for adequate precipitation. DARS was added to separate free PG from antibody-bound PG. After overnight incubation, tubes were centrifuged at 13000 g for 30 min at 4°C. The supernatant was discarded and 2.5 ml of scintillant was added. The tubes were then capped and 'whirlimixed' to re-suspend the pellet, and the radioactivity in each tube was counted in a Packard Liquid Scintillation Counter.

The percentage of [^{3}H]PG bound is calculated by the following formula:

$$\% [^3H]PG \text{ Bound} = \frac{\text{Counts in Tube} \times 100}{\text{Average Std Counts}}$$

where the average standard (Std) counts is the average of the counts in tubes 5-8 (counting standards).

Data from the counter were passed to an IBM-XT personal computer programmed with the Packard Data Acquisition and Analysis system (PC-DAAS). The Spline Curve-Fit programme was used which processes the standards according to a Modified Smooth Spline Algorithm. The non-specific binding from all the standards are subtracted by the programme which then calculates the co-ordinates for a curve of the best fit (Figs. 2.1.6.1.1 - 2.1.6.1.3). The amount of PG in each sample tube is calculated using this curve. The detection limit was 10-30 pg of PG per assay tube.

The intra-assay coefficient of variation was calculated using the formula:

$$\text{Intra-assay coefficient of variation} = \frac{\text{standard deviation of sample duplicate} \times 100}{\text{mean value of sample duplicate}}$$

This coefficient was calculated by the computer for each unknown sample assayed in duplicate. If the coefficient for the sample was greater than 12 %, the sample was re-assayed.

The inter-assay coefficient of variation was calculated using the values obtained from tubes containing a known standard amount of the appropriate PG (0.16 ng/ml

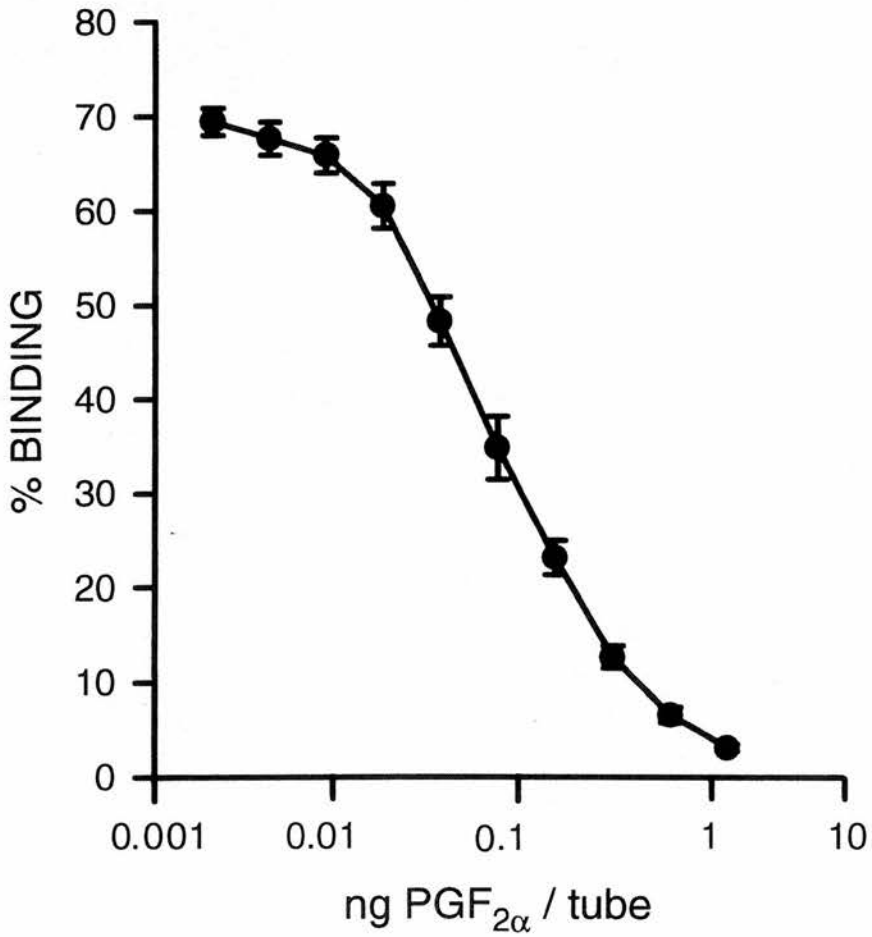


Figure 2.1.6.1.1 Standard curve for $PGF_{2\alpha}$ radioimmunoassay. The data indicate the mean (± SEM, n=6).

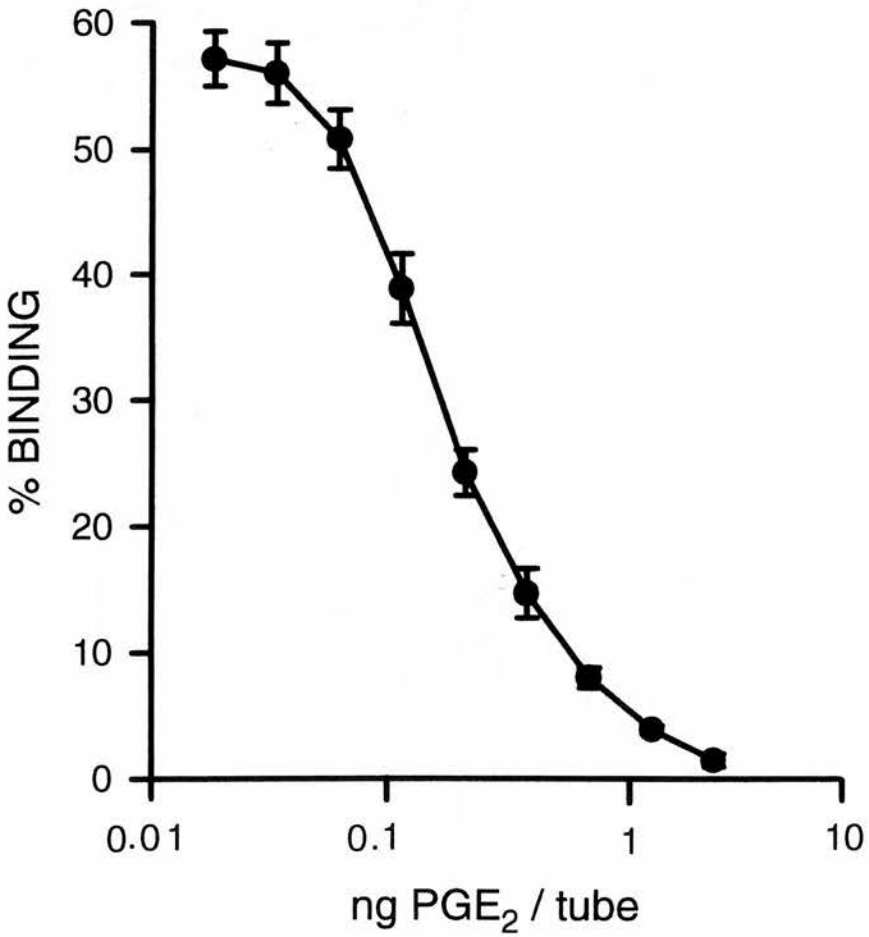


Figure 2.1.6.1.2 Standard curve for PGE_2 radioimmunoassay. The data indicate the mean (± SEM, n=6).

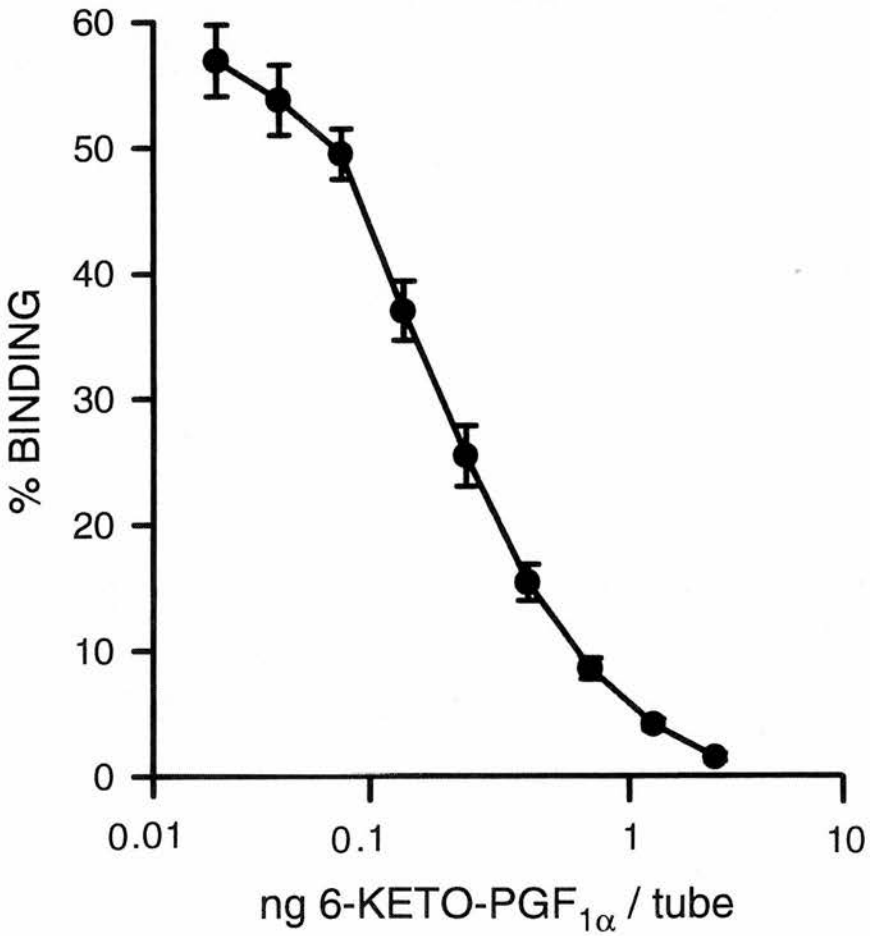


Figure 2.1.6.1.3 Standard curve for 6-KETO-$PGF_{1\alpha}$ radioimmunoassay. The data indicate the mean (± SEM, n=6).

or 0.32 ng/ml), which were included at the end of the assay, according to the formula below:

$$\text{Inter-assay coefficient} = \frac{\text{standard deviation of known PG standard} \times 100}{\text{mean of know PG standard}}$$

This was < 10 % for all PGs measured.

2.1.6.2 Determination of Cross-Reactivities

The cross-reactivities for the prostaglandin antiserum used in these experiments had been determined previously in this laboratory (Dighe *et al.*, 1975; Poyser & Scott, 1980; Lytton & Poyser, 1982). The results of these experiments are shown in Tables 2.1.6.2.1 - 2.1.6.2.3. Standard curves for the PGs and their metabolites were set up. The concentration of PG or PG metabolite that produced a 50 % fall in binding from the binding produced by the zero standard solution of the PG to which the antiserum was raised and the cross-reactivities calculated.

Percentage cross-reactivity was calculated by the following formula:

$$\%\ \text{cross-reactivity} = \frac{\text{concentration PG (to which antisera was raised) giving a 50 \% fall in zero standard binding}}{\text{concentration of different PG or PG metabolite giving a 50 \% fall in zero standard binding}} \times 100$$

The $PGF_{2\alpha}$ and PGE_2 antibodies do not distinguish between the 1- and 2-series prostaglandins. However, previous studies (Poyser, 1983) have shown little PGE_1 and $PGF_{1\alpha}$ to be produced by the guinea-pig uterus. In addition PGA_2 and PGB_2 are not produced naturally and are therefore unlikely to interfere with the PGE_2

Table 2.1.6.2.1 Cross-reactivities with various prostanoids and their metabolites for the $PGF_{2\alpha}$ antiserum (rabbit 5, 6th bleed), measured at 30 % binding of tracer. (TxB_2 is thromboxane B_2).

Prostanoid	% Cross-Reactivity at 30 % binding of tracer
$PGF_{2\alpha}$	100
$PGF_{1\alpha}$	28
PGE_2	0.2
PGE_1	0.5
PGA_2	< 0.1
PGB_2	< 0.1
PGD_2	0.4
15-keto-$PGF_{2\alpha}$	0.5
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.2
6-keto-$PGF_{1\alpha}$	0.5
15-keto-PGE_2	< 0.1
13,14-dihydro-15-keto-PGE_2	0.1
TXB_2	0.6

Table 2.1.6.2.2 Cross-reactivities with various prostanoids and their metabolites for the PGE_2 antiserum (rabbit R5, 6th bleed), measured at 30 % binding of tracer. (TxB_2 is thromboxane B_2).

Prostanoid	% Cross-Reactivity at 30 % binding of tracer
PGE_2	100
PGE_1	94
PGA_2	13.6
PGB_2	72.7
PGD_2	0.4
$PGF_{1\alpha}$	4.3
$PGF_{2\alpha}$	3.8
15-keto-$PGF_{2\alpha}$	0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	<0.1
6-keto-$PGF_{1\alpha}$	0.3
15-keto-PGE_2	0.2
13,14-dihydro-15-keto-PGE_2	0.4
TXB_2	0.2

Table 2.1.6.2.3 Cross-reactivities with various prostanoids and their metabolites for the 6-keto-$PGF_{1\alpha}$ antiserum (rabbit NP1, 6th bleed), measured at 30 % binding of tracer. (TxB_2 is thromboxane B_2).

Prostanoid	% Cross-Reactivity at 30 % binding of tracer
6-keto-$PGF_{1\alpha}$	100
$PGF_{1\alpha}$	0.4
PGE_2	4.2
PGE_1	1.1
PGA_2	< 0.1
PGB_2	< 0.1
PGD_2	< 0.1
$PGF_{2\alpha}$	< 0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.1
15-keto-$PGF_{2\alpha}$	< 0.1
15-keto-PGE_2	0.1
13,14-dihydro-15-keto-PGE_2	0.1
TXB_2	< 0.1

antiserum. Thus the $PGF_{2\alpha}$ and PGE_2 antibodies were removing predominantly the PG to which they were raised.

2.1.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR PGFM

This technique is a variant of RIA in which the label used is an enzyme rather than a radioisotope. The ELISA used here was to measure the amount of prostaglandin $F_{2\alpha}$ metabolite called 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) in culture samples already assayed for PG.

Plates were coated with DARS using direct γ-globulin procedure and were washed four times in wash buffer (for composition see Section 2.2.7e). The first two wells in each plate contained 150 µl ELISA buffer (Section 2.2.7d) and were used to calculate the non-specific binding. The next two wells contained 100 µl ELISA buffer and were used to determine binding only. The final four wells in each assay contained a quality control (QC) with a normal reading of approximately 200 pg/ml.

Ten standard solutions were prepared from a stock solution of 20 µg/ml stored at -20°C. The standards were made up in ELISA buffer and concentrations ranged from 0.01-5.12 ng/ml. These were dispensed in duplicate in 100 µl. Samples were also dispensed in duplicate in 100 µl at the appropriate dilution.

The required dilution of antiserum (1:100) was prepared from a stock solution of 1:50,000 in buffer. Antibodies were raised in the MRC Reproductive Biology Unit, Edinburgh. The label used was a peroxidase conjugate (POX) and a 1:50 dilution was made from a 1:40,000 stock solution. 50 µl of diluted antiserum was added to all

wells except for the first two wells, and 50 µl of POX was added to all wells. The plates were then allowed to incubate overnight at 4°C.

After overnight incubation, the plates were washed several times in wash buffer. Tetramethyl benzidine substrate (TMB) was prepared from stable solutions; 2 ml of urea-peroxidase (0.3g per 50 ml of 100 mM sodium acetate buffer pH 6.0) and 2 ml of tetramethyl benzidine (2 mg/ml in dimethylformamide). These were added to 20 ml of 100 mM sodium acetate buffer (pH 6.0). TMB substrate (200 µl) was added to all wells and the plates were left for approximately 10 min to allow colour to develop. After this time, 50 µl of 2N sulphuric acid was added to quench development of colour. The plates were read in a labsystems multiskan MCC/340 plate-reader and the results calculated on an assayzap program.

For this ELISA the inter-assay coefficient of variation was calculated as 14.7 % while the intra-assay coefficient of variation was calculated as 6.8 % (see Section 2.1.6.1).

The cross-reactivities for the antiserum used in these experiments had been determined previously in the Reproductive Biology Unit laboratory, Edinburgh (Kelly *et al.*, 1986). The results of these experiments are shown in Table 2.1.7.1.

2.1.8 STATISTICS

Statistical methods are used to analyse experimental results so that they can be displayed in a way that is easy to comprehend. The arithmetic mean or the simple 'mean' of a population is usually the first thing calculated, otherwise known as the 'most typical value'. This is the same as the average. The mean is the summation of the individual values divided by the number of values or the (n) number. The aim is

Table 2.1.7.1 Cross-reactivities with various prostanoids and their metabolites for the 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) antiserum.

Prostanoid	% Cross-Reactivity tracer
13,14-dihydro-15-keto-$PGF_{2\alpha}$	100
PGE_2	<0.02
6-keto-$PGF_{1\alpha}$	0.01
PGD_2	<0.01
13,14-dihydro-15-keto-PGE_2	0.19
TXB_2	< 0.01

to determine whether the hypothesis that the samples studied were drawn from the same population is true. When there is more than two samples to compare, then a multidimensional *t*-test is used which is called the analysis of variance (ANOVA) but, while this test does tell you whether the groups are significantly different, it doesn't tell you which groups differ from each other.

The purpose of the *t*-test is to assess the significance of the difference in the means of two groups (e.g. the difference between a control group and a treated group). Before calculating the value of *t*, the means and standard deviations of the two groups being studied are required. The preconditions of doing a *t*-test are that the distribution of the data should be normal, the number of groups to be compared should be two and that there should be no significant difference in the variance of the two groups being compared. The F-test is used to determine this and the formula for this is shown below:

$$\text{F test} = \frac{\text{population variance estimated from sample mean}}{\text{population variance estimated as average of the sample variance}}$$

The difference between the *t*-test and the F-test is that the *t*-test compares the means while the F-test compares variance (the square of the standard deviation). If the hypothesis that all the samples in each experiment are drawn from the same population is true, then the within group variance and between group variance are both estimates of the same population variance and so should be about equal (Glantz, 1992). If 'F' is a big number then the variability between samples is larger than expected from the variability within the samples and the hypothesis that the samples

are drawn from the same population is rejected. If there is no significant difference between standard deviations then we can go ahead and calculate the value of '*t*'.

The formula for the *t*-test is shown below:

$$t\text{-test} = \frac{\text{Difference in sample means}}{\text{Standard error of difference of sample means}}$$

Values for '*t*' and 'F' are tabulated and depend on two factors. The first of these is the number of samples in each experiment otherwise known as degrees of freedom (d.f.). The greater the sample size, the more likely it is that there will be a significant difference in the population. The d.f. is calculated by n-1 (where n is the number of samples in each group). As the sample size increases then the value of '*t*' required to reject the hypothesis that the samples came from the same population decreases. The second factor is the level of confidence with which one rejects the hypothesis of no difference. This is known as the P value, which stands for probability, and is expressed either as a percentage or as a decimal. As the value of '*t*' increases, then the P value decreases and thus becomes more significant.

'Duncan's Multiple Range Test' was used to test for significant differences of increasing size in more than 2 samples analysed at the same time. In this test the confidence levels are not appropriate and are replaced by 'protection levels' against finding false significant differences. It uses a variable level depending on the number of means involved at each stage. The idea is that as the number of means under test increases, the smaller is the probability that they will be alike.

To summarise, since the interest was on the mean, which gives more information about the samples tested, and the data were a continuous variable, the students t-test

was therefore used. Where this was not appropriate Duncan's multiple range test was used to determine if the hypothesis that all the samples originate from a single population was true.

2.2 MATERIALS

The sources of the materials are as follows:

2.2.1 Solvents

Acetonitrile - Rathburn Chemicals Ltd, Walkerburn, Scotland, UK.

Ethyl acetate* - Prolabo, 54 rue Roger Salengro, 94120, Fontenay, S/Bois.

2-Ethoxyethanol - Fisher Scientific UK Ltd, Leicestershire, UK.

Methanol - Rathburn Chemicals Ltd, Walkerburn, Scotland, UK.

Toluene - Fisher Scientific UK Ltd, Leicestershire, UK.

* Redistilled before used

2.2.2 Chemicals

Actinomycin D - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Adenosine - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Amphotericin B - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

ATP (Adenosine 5'-triphosphate) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

α,β Methylene ATP - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

β,γ Methylene ATP - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Calcium chloride - B.D.H. Laboratory Supplies, Poole, UK.

Cycloheximide - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

D-Glucose - B.D.H. Laboratory Supplies, Poole, UK.

EGTA (Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid) -

Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Gonadotrophin releasing hormone - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Hydrochloric acid - B.D.H. Laboratory Supplies, Poole, UK.

Indomethacin - Merck, Sharpe & Dohme Ltd, Hoddesdon, Herts, UK.

Kanamycin - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

L-Glutamine - ICN, Oxfordshire, UK.

Magnesium sulphate – B.D.H. Laboratory Supplies, Poole, UK.

Medium 199 - ICN, Oxfordshire, UK.

2-Methylthio ATP - RBI, St Albans, Herts., UK.

Nifedipine - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methanesulphonamide) - Calbiochem, Nottingham, UK.

Potassium chloride - Fisons Scientific Equipment, Loughborough, UK.

PPO (2,5-Diphenyloxazole) - Fisher Scientific UK Ltd, Leicestershire, UK.

Puromycin - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Sodium chloride - Fisher Scientific UK Ltd, Leicestershire, UK.

Sodium hydrogen carbonate – B.D.H. Laboratory Supplies, Poole, UK.

8-Sulphophenyltheophylline - RBI, St Albans, Herts., UK.

Suramin - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

TFP (Trifluoperazine) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

TMB-8 (3,4,5 Trimethoxybenzoic acid 8-(diethylamino) octyl ester hydrochloride) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

TPA (Phorbol 12 myristate 13-acetate) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

UTP (Uridine triphosphate) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Verapamil - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide) - Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

2.2.3 Radioactive Compounds

[5, 6, 8, 9, 11, 12, 14, 15 (n) - ^{3}H] $PGF_{2\alpha}$

[5, 6, 8, 11, 12, 14, 15 (n) - ^{3}H] PGE_2

[5, 8, 9, 11, 12, 14, 15 (n) - ^{3}H] 6-Keto-$PGF_{1\alpha}$

All [^{3}H]PG stock solutions were diluted to 5 μCi/ml and stored at -20°C before being diluted further and used for radioimmunoassay. [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 were initially diluted in methanol and [^{3}H]6-keto-$PGF_{1\alpha}$ was diluted in a 1:9 mixture of acetonitrile and water. All radioactive compounds were supplied by Amersham International Ltd, Cardiff, UK.

2.2.4 Antibodies

Normal rabbit serum (NRS) and donkey anti-rabbit serum (DARS) - Scottish Antibody Production Unit, Carluke, Scotland, UK.

$PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ antibodies were all raised in this laboratory in rabbits (Dighe *et al.*, 1975; Dighe *et al.*, 1978a; Dighe *et al.*, 1978b).

2.2.5 Gases

95% O_2 : 5% CO_2 - British Oxygen Co. Ltd., Guildford, UK

2.2.6 Other Materials

Plastic tubes (3 ml) and caps - Greiner, Gloucestershire, UK

Easy grip tissue culture dishes (sterile/gamma irradiated) - Becton Dickinson, Plymouth, UK

Plastic blood containers (5 ml) - Sterelin Ltd, Teddington, UK

Stainless steel mesh - Advent Research Materials, Suffolk, UK

2.2.7 Composition of Solutions

(a) Tris Diluent (0.05M, pH 8.0) used for $PGF_{2\alpha}$ assay

Tris	30.25 g
NaN_3	0.5 g
H_2O	5 L

Add 12 ml concentrated HCl and check pH

(b) Tris Diluent (0.05M, pH 6.8) used for 6-keto-$PGF_{1\alpha}$ assay

Tris	30.25 g
NaN_3	0.5 g
H_2O	5 L

Add 14.5 ml concentrated HCl and check pH

(c) Phosphate Diluent (0.05M, pH 7.5) used for PGE_2 assay

$NaHPO_4$ (anhydrous)	34.5 g
H_2O	5 L
1M $NaH_2PO_42H_2O$	56 ml
NaN_3	0.5 g

Check pH

(d) ELISA Buffer (pH 7.2) used for PGFM assay

100 mM Tris pH 7.2

150 mg/litre 2-methylisothizolone

150 mg/litre Bromo nitro dioxane

2 mg/ml BSA

Phenol red 300 µl 0.5 % solution per litre

Sodium chloride 9 g/litre

EDTA 2 mM (4 ml/litre of 0.5 M stock)

Check pH

(e) ELISA wash buffer used for PGFM assay

1 cap full of ELISA buffer

0.5 L water

(f) Krebs' Solution

NaCl	34.5 g
D-Glucose	10.5 g
$NaHCO_3$	10.5 g
10% KCl solution	17.7 ml
10% $MgSO_4.7H_2O$ solution	14.5 ml
10% KH_2PO_4 solution	8.0 ml
M $CaCl_2$ solution	12.6 ml

Make up to 5 L with H_2O

(g) Scintillant

Toluene	1.5 L
2,5-Diphenyloxazole (PPO)	10.5 g
2-ethoxyethanol	0.9 L

SECTION THREE

3. RESULTS

This section deals, in full detail, with all the results obtained from the experiments carried out for this thesis.

3A. AN INVESTIGATION INTO THE EFFECT OF ADENOSINE 5'-TRIPHOSPHATE (ATP) ON PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG UTERUS.

Introduction:

Previously it has been observed that adenosine and adenine nucleotides interact with prostaglandins (PGs) in a number of tissues (Dozi-Vassilides *et al.*, 1976) and in particular cause contraction of the guinea-pig uterus (Moritoki *et al.*, 1979; Smith *et al.*, 1988; Scheimann *et al.*, 1991; Piper & Hollingsworth, 1996). Piper & Hollingsworth (1996) have shown that adenosine 5′-triphosphate (ATP) and its analogues contract the non-pregnant guinea-pig uterus *in vitro*. These spasmogenic actions were inhibited by the prostaglandin H synthase (PGHS) inhibitor indomethacin suggesting that PGs mediate this contractile effect (Piper & Hollingsworth, 1996). Therefore, the effects of ATP, 2 methylthio-ATP, αβ methylene-ATP, βγ methylene-ATP, UTP and adenosine on PG production by the guinea-pig uterus have been investigated. Studies were carried out on non-pregnant

guinea-pig uterus superfused *in vitro* and, on guinea-pig endometrium and myometrium cultured for a period of 24 h.

These agonists act at adenosine (A, formerly P1) receptors and P2 purinoceptors. ATP acts via P2 purinoceptors and, adenosine via A receptors. However, ATP can be hydrolysed by ecto-nucleotidases and metabolised to adenosine (Paton, 1985), so it could exert its effect via P2 and/or A receptors. P2 and A receptors have previously been identified in the guinea-pig myometrium by Smith *et al.* (1988) and this group have shown that ATP-induced contraction of guinea-pig myometrium is mediated by both P2 and A receptors.

In this study the effects of an A receptor antagonist, 8-sulphophenyltheophylline, and a P2 antagonist, suramin, have also been investigated on the uterus superfused *in vitro*, and on the endometrium and myometrium cultured for 24 h. 8-Sulphophenyltheophylline and suramin are non-selective A and P2 antagonists, respectively. These studies were carried out in the presence of ATP and adenosine. This was to determine if the prostaglandin-releasing effects of ATP were mediated via P2 purinoceptors or whether, after being broken down to adenosine, ATP could act via adenosine receptors. Total PG production by the guinea-pig uterus was also measured, using endometrial and myometrial homogenates, after the tissue had been cultured for a period of 24 h with ATP or adenosine, either on their own or in the presence of suramin (a P2 receptor antagonist) or 8-sulphophenyltheophylline (an A receptor antagonist).

Methods:

For superfusion experiments, the guinea-pig uterus was removed on day 7 of the oestrous cycle as described previously (Section 2.1.1), separated into 2 uterine horns, blotted dry and weighed. Each horn was cut longitudinally along the anti-mesometrial side and suspended in an organ bath with one end attached to an isotonic lever. Each uterine horn was superfused independently with Krebs solution at 5 ml/min at 37°C for a 60 min equilibration period. Samples where collected every 10 min for the next 80 min (8 samples per uterine horn) or 100 min (10 samples per uterine horn). One of the uterine horns was untreated and acted as a control. The test uterine horn was superfused with either ATP (100 μM), 2 methylthio-ATP (1 μM), αβ methylene-ATP (100 μM), βγ methylene-ATP (100 μM), UTP (100 μM) or adenosine (100 μM) between 30 and 50 min after equilibration (samples 4 and 5) (for experiment 3A.1). In experiment 3A.3, the test uterine horn was treated with suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) between 30-50 min after equilibration (samples 4 and 5), followed by treatment with either suramin (100 μM) plus ATP (100 μM) or adenosine (100 μM), or with 8-sulphophenyltheophylline (140 μM) plus ATP (100 μM) or adenosine (100 mM) between 50-70 min after equilibration (samples 6 and 7). Following treatment with the appropriate drug, all samples were treated in the same way. The pH of each sample was lowered to 4.0 and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate, according to the method described by Poyser (1972). The two ethyl acetate fractions were combined and evaporated to dryness on a rotary evaporator. Samples were re-dissolved in 10 ml ethyl acetate and stored at -20°C before assaying for their $PGF_{2\alpha}$,

PGE_2 and 6-keto-$F_{1\alpha}$ content. Changes in PG output with time were analysed by the Duncan's multiple range test.

Culture experiments were carried out using the guinea-pig uterus on day 7 of the oestrous cycle as described in Section 2.1.2. The endometrium and myometrium were separated and prepared for culture as described in Section 2.1.2. The tissue was cultured separately on raised platforms in Petri dishes containing tissue culture medium (see Section 2.1.2). Kilner jars contained 8 Petri dishes (2 controls and 6 treated). The endometrium and myometrium in experiment 3A.2 were treated with either ATP (10, 50 and 100 μM), 2 methylthio-ATP (1, 5, 10 and 25 μM), αβ methylene-ATP (10, 50 and 100 μM), βγ methylene-ATP (10, 50 and 100 μM), UTP (10, 50 and 100 μM) or adenosine (10, 50 and 100 μM). In experiment 3A.4, the endometrium and myometrium were treated with ATP (100 μM), ATP (100 μM) plus suramin (100 μM), ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM), adenosine (100 μM), adenosine (100 μM) plus suramin (100 μM), or adenosine (100 μM) plus 8-sulphophenyltheophylline (140 μM). All samples were collected after 2, 8 and 24 h and stored at -20°C before being assayed for their PG content. The results from these experiments were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

PG production by homogenates of guinea-pig endometrium and myometrium was measured using the tissue after 24 h culture with ATP (100 μM), adenosine (100 μM), and ATP (100 μM) or adenosine (100 μM) with either suramin (100 μM) or 8-

sulphophenyltheophylline (140 μM). Control, untreated tissues were also used. The tissue was blotted dry, weighed and then homogenised as described in Section 2.1.3. Each sample was incubated for 60 min in a water bath at 37°C and 230 oscillations/min. The pH of each sample was lowered to 4.0 using 1 M HCl, and prostaglandins were extracted as described in Section 2.1.1. The extracts were evaporated to dryness on a rotary evaporator. Samples were re-dissolved in 5 ml ethyl acetate and were stored at -20°C until assayed for their PG content. The results from these experiments were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

Experiment 3A.1 The Effects of ATP, Adenosine and ATP Analogues on Prostaglandin Output From Day 7 Guinea-Pig Uterus Superfused *In Vitro.*

ATP (100 μM), 2 methylthio-ATP (1 μM), UTP (100 μM) and adenosine (100 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output from the day 7 guinea-pig uterus superfused *in vitro* (Figs. 3A.1.1a, 3A.1.3a, 3A.1.5a and 3A.1.6a). αβ Methylene-ATP (100 μM) and βγ methylene-ATP (100 μM) had no significant effect on $PGF_{2\alpha}$ output (Figs. 3A.1.2a and 3A.1.4a). PGE_2 output from guinea-pig uterus was unaffected by ATP (100 μM), αβ methylene-ATP (100 μM), 2 methylthio-ATP (1 μM), βγ methylene-ATP (100 μM), UTP (100 μM) and adensoine (100 μM) (Figs. 3A.1.1b-3A.1.6b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) increased

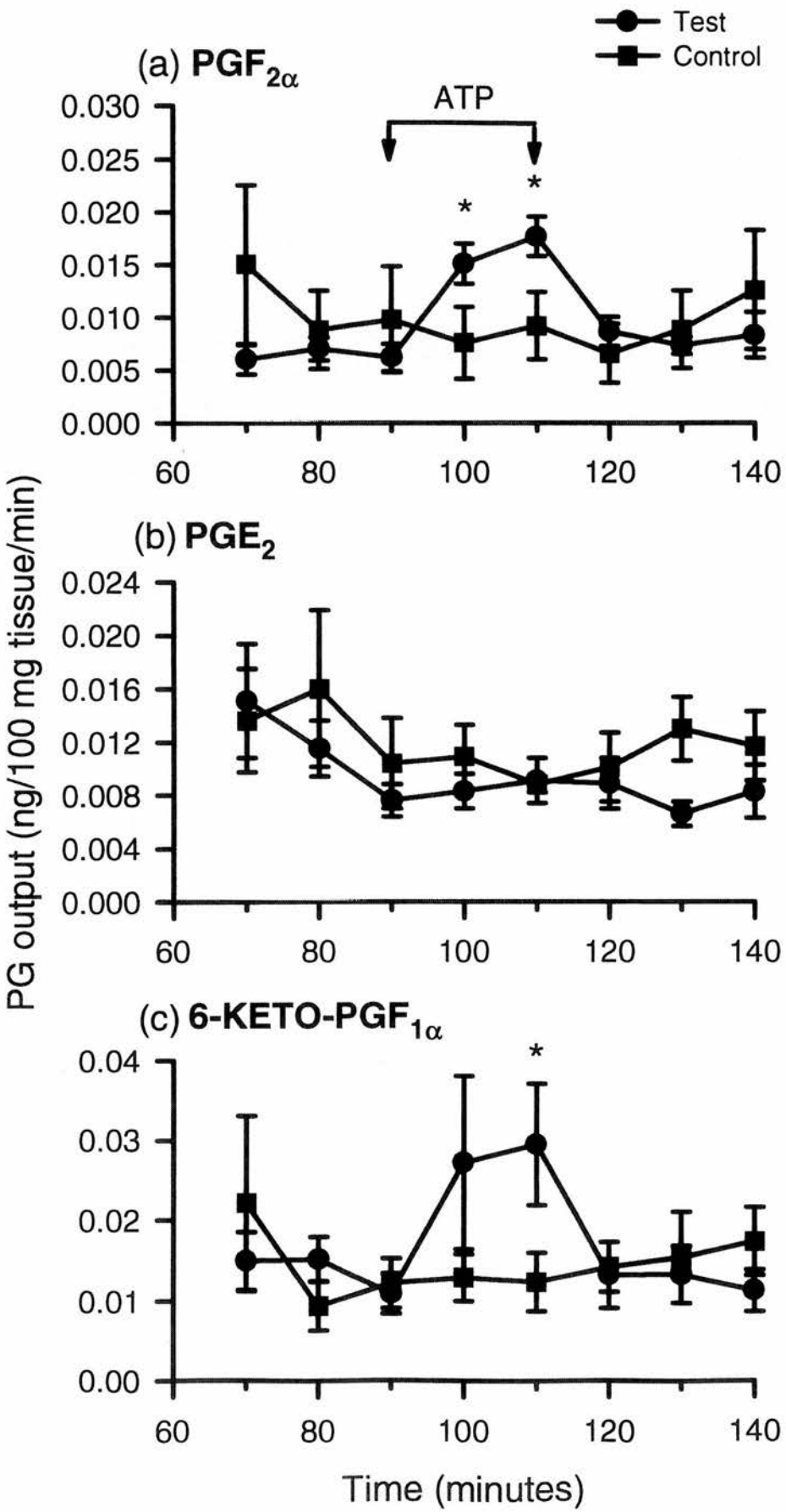


Figure 3A.1.1 Effect of ATP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding ATP treatment.

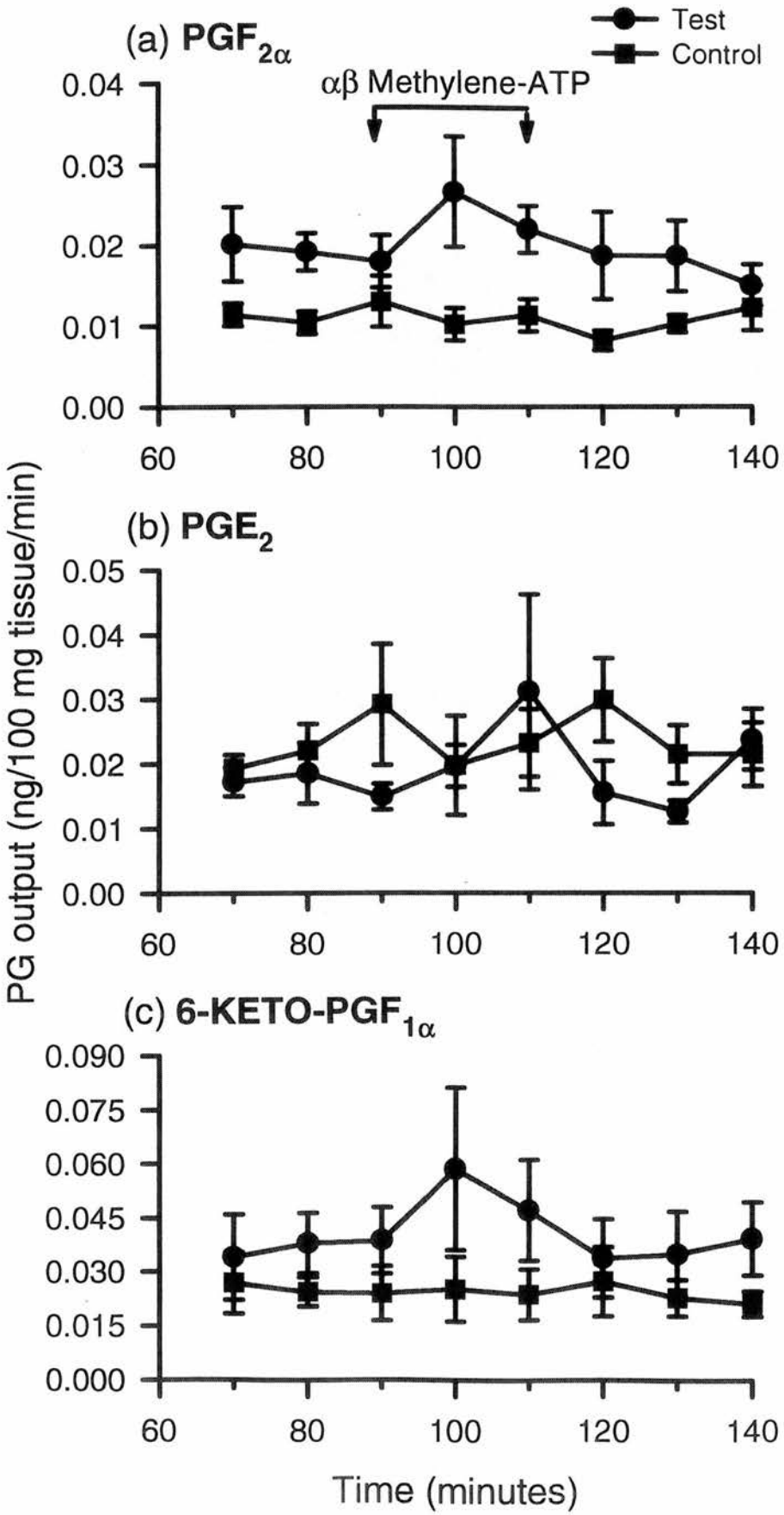


Figure 3A.1.2 Effect of αβ methylene-ATP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.

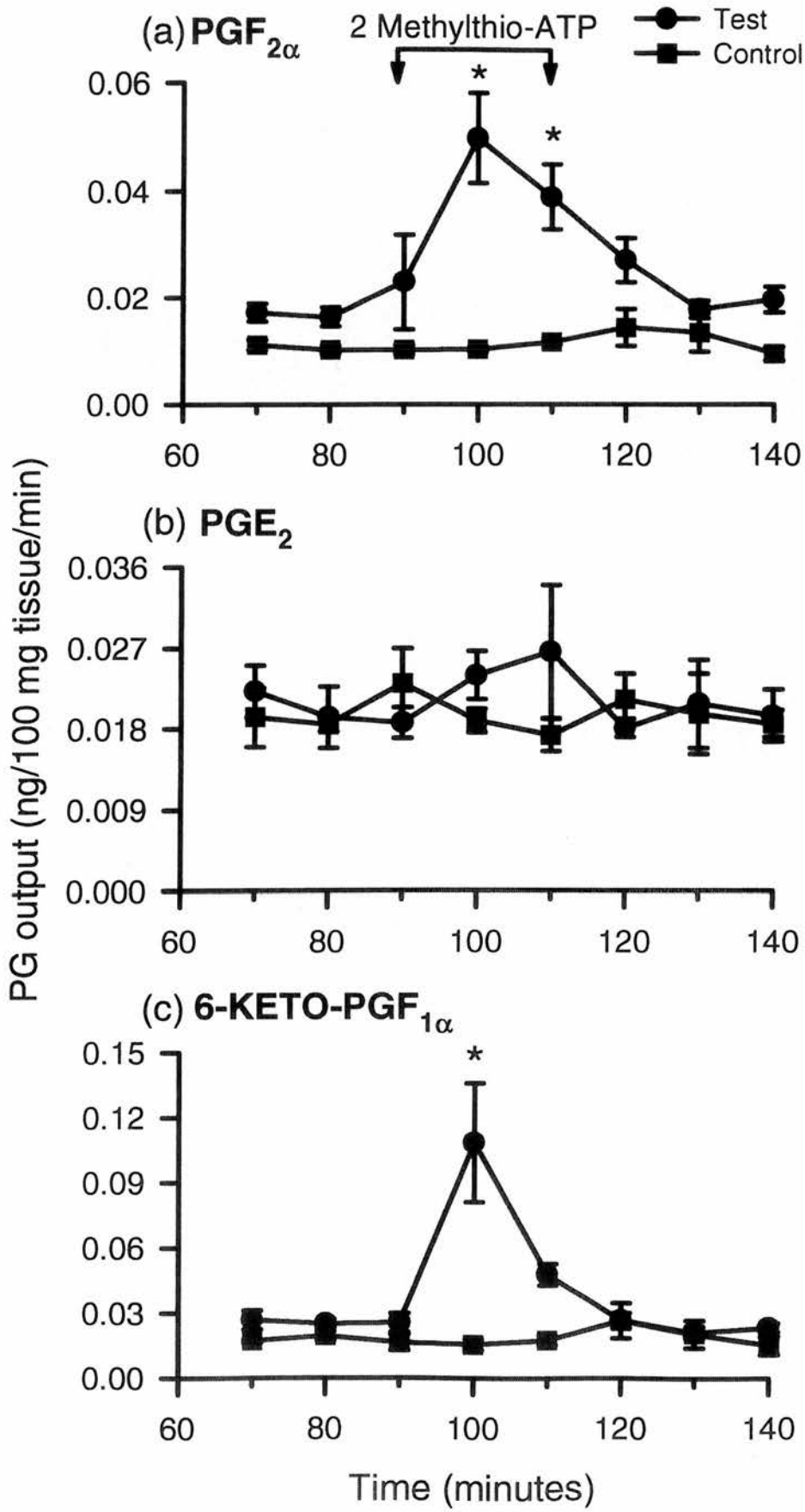


Figure 3A.1.3 Effect of 2-methylthio-ATP (1 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding 2 methylthio-ATP treatment.

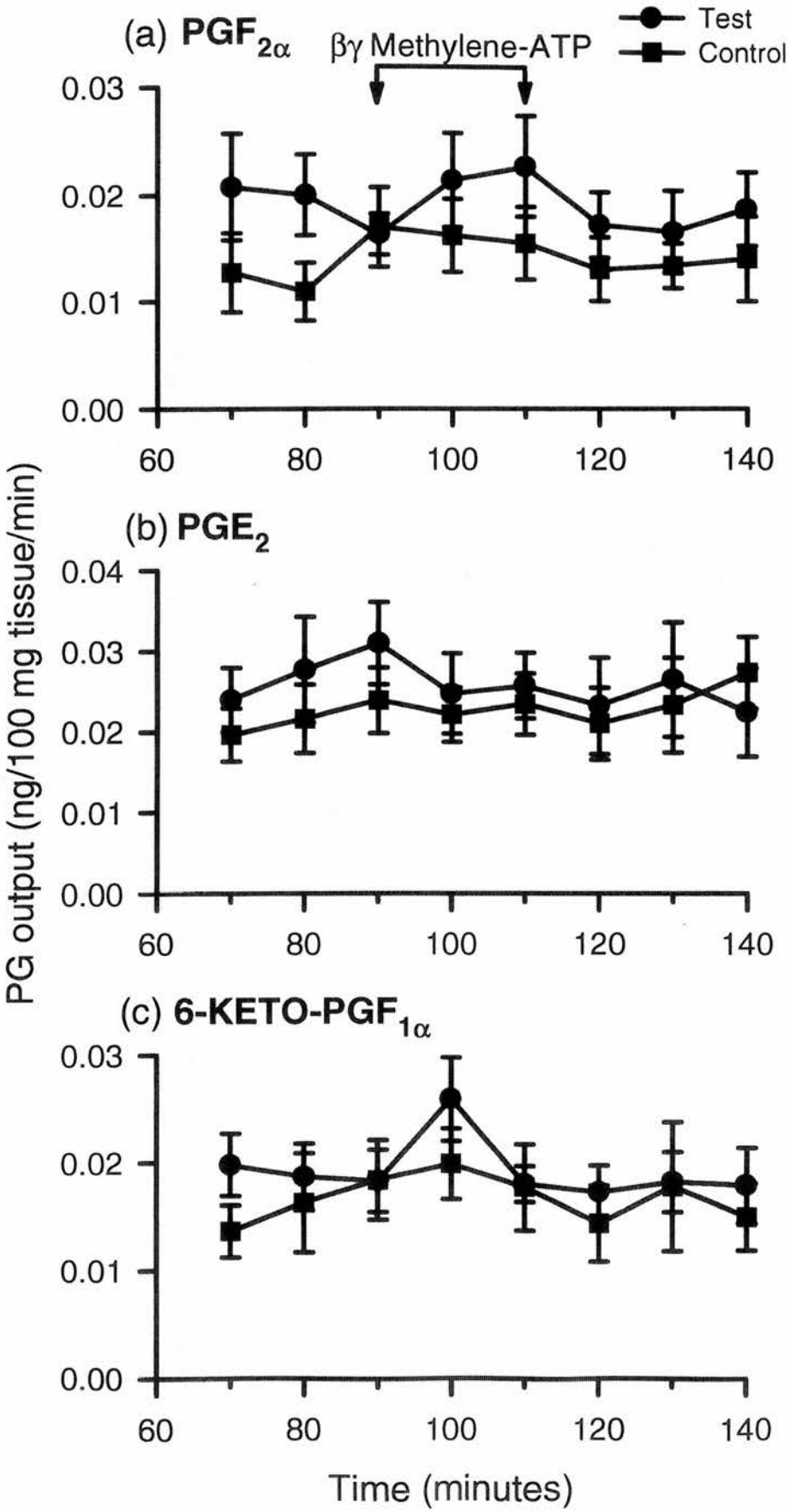


Figure 3A.1.4 Effect of βγ methylene ATP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.

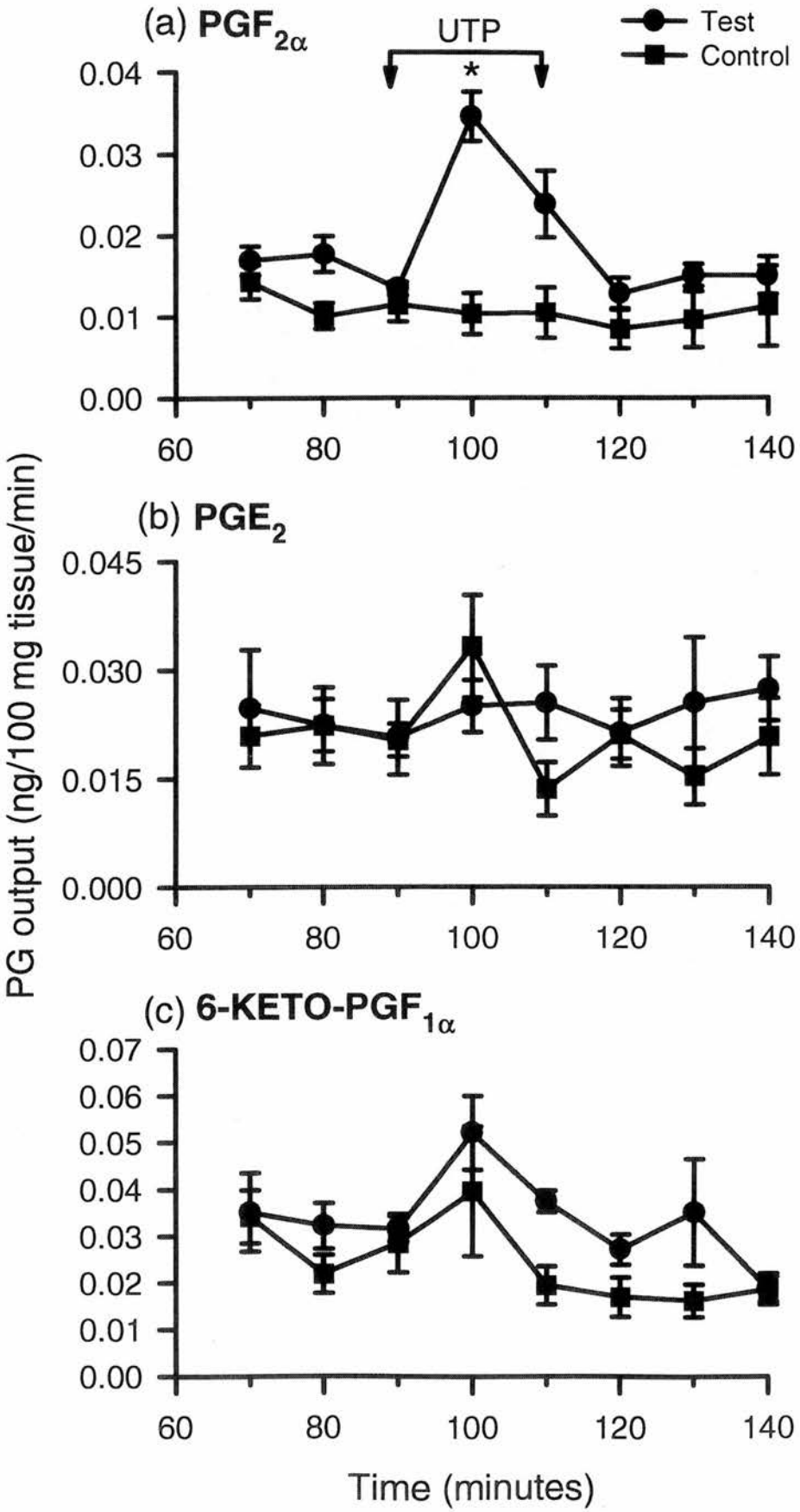


Figure 3A.1.5 Effect of UTP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding UTP treatment.

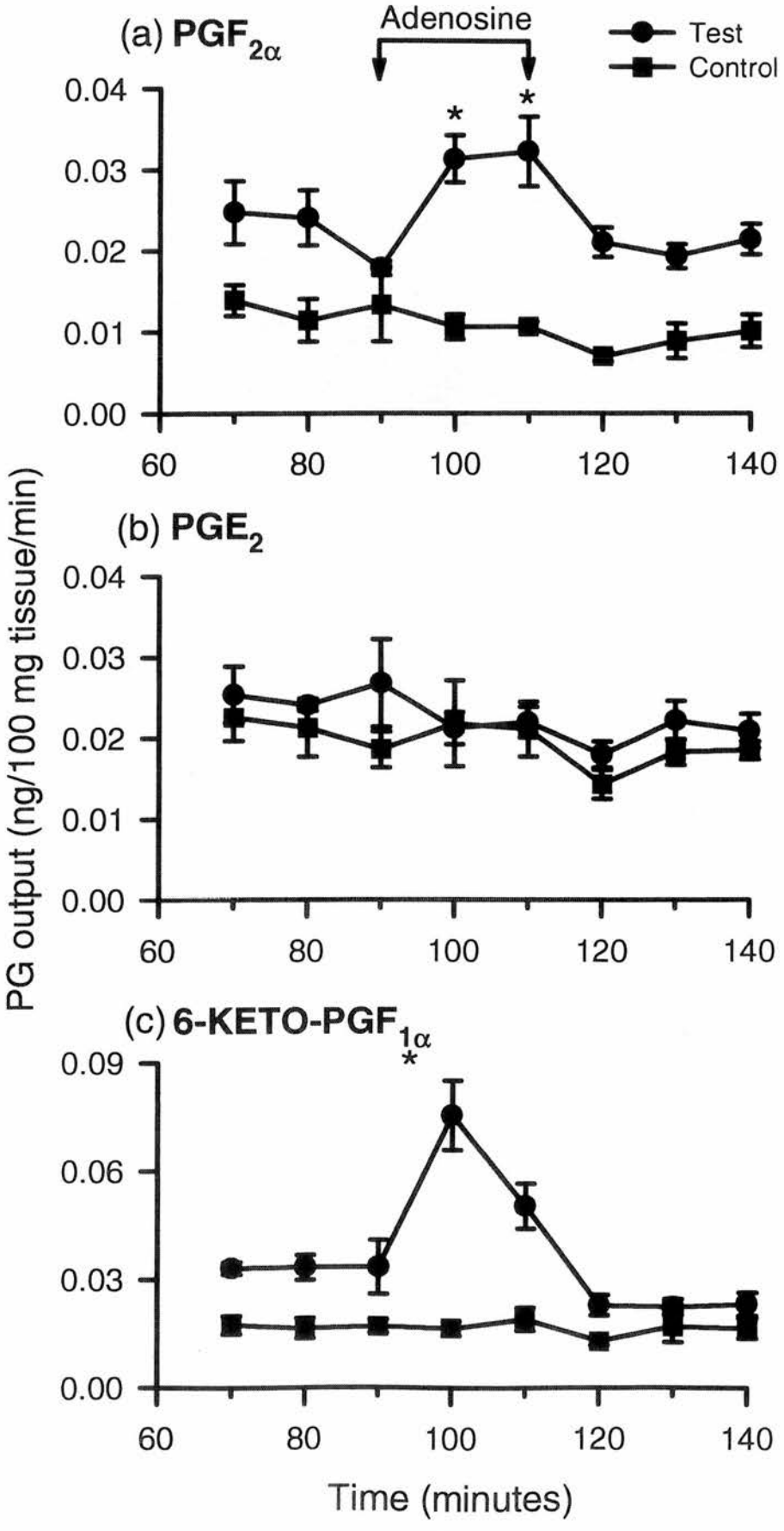


Figure 3A.1.6 Effect of adenosine (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding adenosine treatment.

by ATP (100 μM), 2 methylthio-ATP (1 μM) and adenosine (100 μM) (Figs. 3A.1.1c, 3A.1.3c and 3A.1.6c). αβ Methylene-ATP (100 μM), βγ methylene-ATP (100 μM) and UTP (100 μM) had no significant effect on the outputs of 6-keto-$PGF_{1\alpha}$ (Figs. 3A.1.2c, 3A.1.4c and 3A.1.5c).

Experiment 3A.2 The Effects of ATP, Adenosine and ATP Analogues on Prostaglandin Output from Day 7 Guinea-Pig Endometrium and Myometrium in Culture.

Endometrium

ATP (10, 50 and 100 μM) and 2 methylthio-ATP (1, 5, 10 and 25 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ outputs from guinea-pig endometrium after 24 h of culture but had no effect after 2 h and 8 h of culture (Figs. 3A.2.1a and 3A.2.3a). ATP (10, 50 and 100 μM) had no significant effect on PGE_2 output from guinea-pig endometrium (Fig. 3A.2.1b). 2 Methylthio-ATP (1, 5, 10 and 25 μM) had no effect on PGE_2 output, except for 5 μM 2 methylthio-ATP which significantly ($P < 0.05$, n=5) decreased PGE_2 output after 8 h of culture and 1 μM 2 methylthio-ATP which significantly ($P < 0.05$, n=5) increased PGE_2 output after 24 h (Figs. 3A.2.1b and 3A.2.3b). ATP (10, 50 and 100 μM) and 2 methylthio-ATP (5, 10 and 25 μM) significantly ($P < 0.05$, n=5) decreased 6-keto-$PGF_{1\alpha}$ output after 8 h and 24 h of culture, but had no effect during the first 2 h of culture (Fig. 3A.2.1c). 2 Methylthio-

ATP (1 μM) had no significant effect on 6-keto-$PGF_{1\alpha}$ output from endometrium cultured for 24 h (Fig. 3A.2.3c).

αβ Methylene-ATP (10, 50 and 100 μM) had no effect on $PGF_{2\alpha}$ output after 2 h of culture but significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output after 8 h and 24 h, except for the 10 μM concentration after 8 h (Fig. 3A.2.3a). αβ Methylene-ATP (10, 50 and 100 μM) significantly increased PGE_2 output after 24 h but not after 2 and 8 h of culture (Figs. 3A.2.3a and b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) decreased by αβ methylene-ATP (50 and 100 μM, but not by 10 μM) after 8 h of culture (Fig. 3A.2.3c). αβ Methylene-ATP had no effect on 6-keto-$PGF_{1\alpha}$ output after 2 h and 24 h (Fig. 3A.2.3c).

βγ Methylene-ATP (10, 50 and 100 μM) had no significant effect on $PGF_{2\alpha}$ output after 2 h of culture but stimulated $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5) after 8 and 24 h, except for 10 μM βγ methylene-ATP after 8 h (Fig. 3A.2.5a). PGE_2 output was significantly ($P < 0.05$, n=5) increased by βγ methylene-ATP (10, 50 and 100 μM) after 24 h of culture but had no effect after 2 h and 8 h of culture, except 50 μM βγ methylene-ATP which decreased PGE_2 output significantly in the first 2 h ($P < 0.05$, n=5) (Fig. 3A.2.5b). 6-Keto-$PGF_{1\alpha}$ output was not affected by βγ methylene-ATP except, after 24 h, 6-keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) increased by βγ methylene-ATP (50 μM) (Fig.3A.2.5c).

Adenosine (10, 50 and 100 μM) and UTP (10, 50 and 100 μM) significantly ($P < 0.05$, n=5) stimulated $PGF_{2\alpha}$ output after 24 h of culture, but not after 2 and 8 h (Fig. 3A.2.7a). PGE_2 output was significantly ($P < 0.05$, n=5) reduced by adenosine (50 and 100 μM, but not 10 μM) and by UTP (10 μM, but not 50 and 100 μM) in the

first 2 h of culture (Fig. 3A.2.7b). Adenosine (10, 50 and 100 μM) and UTP (10, 50 and 100 μM) had no effect on PGE_2 output after 8 and 24 h of culture, except UTP (100 μM) which significantly ($P < 0.05$, n=5) decreased PGE_2 output after 8 h and adenosine (100 μM) which significantly ($P < 0.05$, n=5) increased PGE_2 output after 24 h of culture (Fig. 3A.2.7b). Adenosine (10, 50 and 100 μM) and UTP (10, 50 and 100 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output, except adenosine (10 and 50 μM) which increased 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$) after 24 h of culture (Fig. 3A.2.7c).

Myometrium

ATP (10, 50 and 100 μM) and 2 methylthio-ATP (1, 5, 10 and 25 μM) had no effect on $PGF_{2\alpha}$ output except, after 2 h, $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) increased by 5 μM 2 methylthio-ATP and, after 24 h, by 1 and 25 μM 2 methylthio-ATP (Figs. 3A.2.2a and 3A.2.4a). PGE_2 output was significantly ($P < 0.05$, n=5) reduced by ATP (10 and 100 μM, but not 50 μM) throughout the 24 h culture period, except by the 10 μM concentration after 8 h of culture (Fig. 3A.2.2b). 2 Methylthio-ATP (1 μM, but not 5, 10 and 100 μM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output after 2 h but not after 8 and 24 h of culture (Figs. 3A.2.2b and 3A.2.4b). 2 Methylthio-ATP (5 and 10 μM, but not 1 and 25 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output after 8 and 24 h of culture (Figs. 3A.2.2b and 3A.2.4b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) reduced by 2 methylthio-ATP (1 μM) after the first 2 h of culture but not after 8 and 24 h of culture (Fig. 3A.2.4c). ATP (10, 50 and 100 μM) and 2 methylthio-ATP (5, 10 and 25 μM) had no effect on 6-

keto-$PGF_{1\alpha}$ output after 2 h of culture (Fig. 3A.2.2c). ATP (10, 50 and 100 μM) and 2 methylthio-ATP (5, 10 and 25 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h (Fig. 3A.2.2c).

αβ Methylene-ATP (10, 50 and 100 μM) had no effect on $PGF_{2\alpha}$ output after 2 h and 8 h of culture, except for 50 μM αβ methylene-ATP which increased $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5) after 8 h (Fig. 3A.2.4a). αβ Methylene-ATP (10, 50 and 100 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output after 24 h (Fig. 3A.2.4a). αβ Methylene-ATP (10, 50 and 100 mM) had no significant effect on PGE_2 output throughout the 24 h culture period (Fig. 3A.2.4b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) increased by αβ methylene-ATP (50 μM, but not 10 and 100 μM) in the first 2 h of culture and reduced by αβ methylene-ATP (10 and 50 μM, but not 100 μM) after 24 h (Fig. 3A.2.4c).

$PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) increased by βγ methylene-ATP (50 μM, but not 10 and 100 μM) after 2 h of culture, by βγ methylene-ATP (10 and 50 μM, but not 100 μM) after 8 h of culture and by βγ methylene-ATP (10, 50 and 100 μM) after 24 h of culture (Fig. 3A.2.6a). βγ Methylene-ATP (10, 50 and 100 μM) significantly ($P < 0.05$, n=5) increased PGE_2 output after 24 h (Fig. 3A.2.6b). PGE_2 output was reduced significantly ($P < 0.05$, n=5) by βγ methylene-ATP (10 and 100 μM, but not 50 μM) after 2 h (Fig. 3A.2.6b). βγ methylene-ATP had no effect on PGE_2 output after 8 h of culture (Fig. 3A.2.6b). 6-Keto-$PGF_{1\alpha}$ output was not significantly affected by βγ methylene-ATP throughouth the 24 h culture period (Fig. 3A.2.6c). UTP (10, 50 and 100 μM) and adenosine (10 and 100 μM, but not 50 μM) increased $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5) after 24 h of culture but not

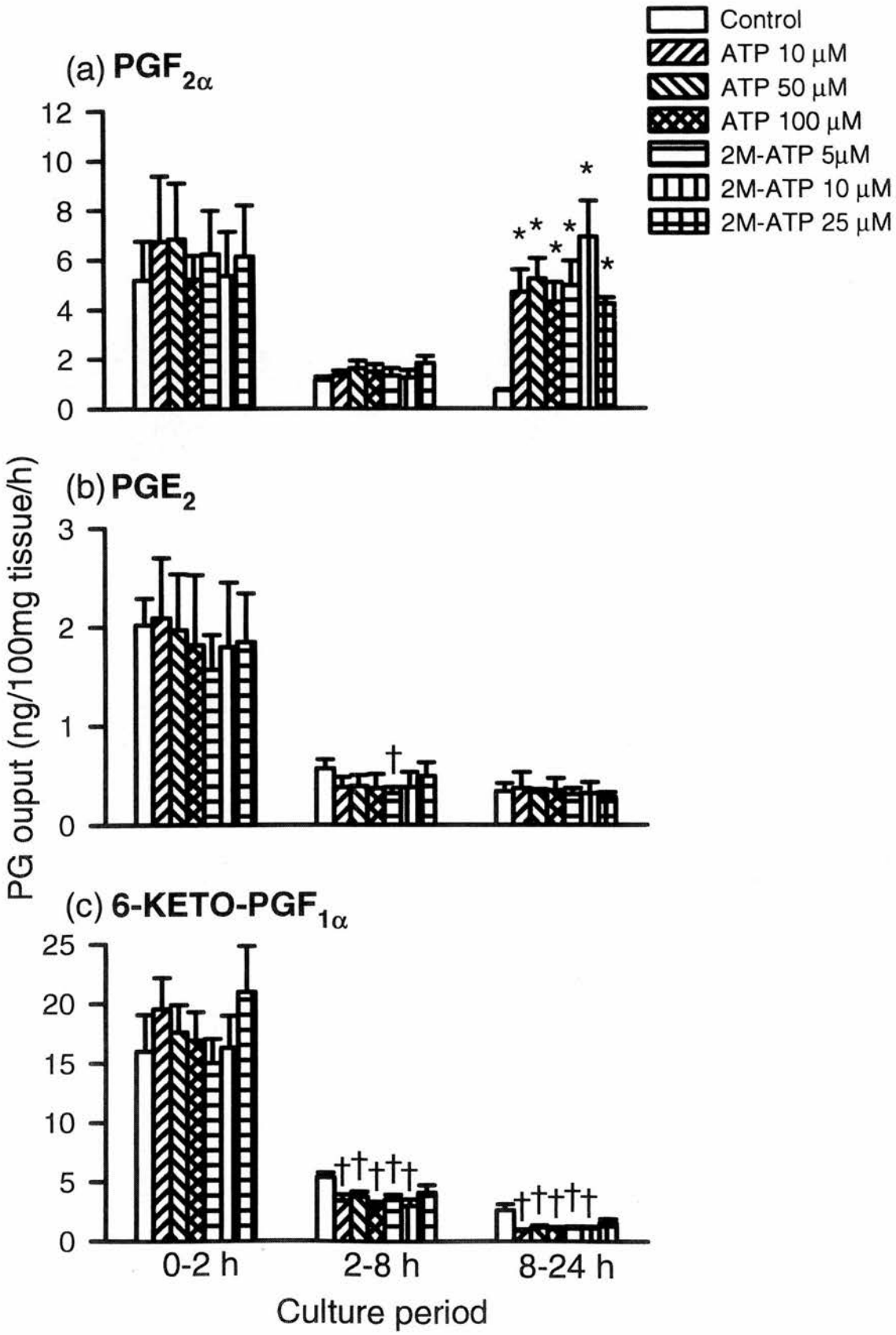


Figure 3A.2.1 Effects of ATP (10, 50 and 100 μm) and 2 methylthio-ATP (5, 10 and 25 μm) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.
*/† Significantly higher/lower, $P < 0.05$, than corresponding control value.

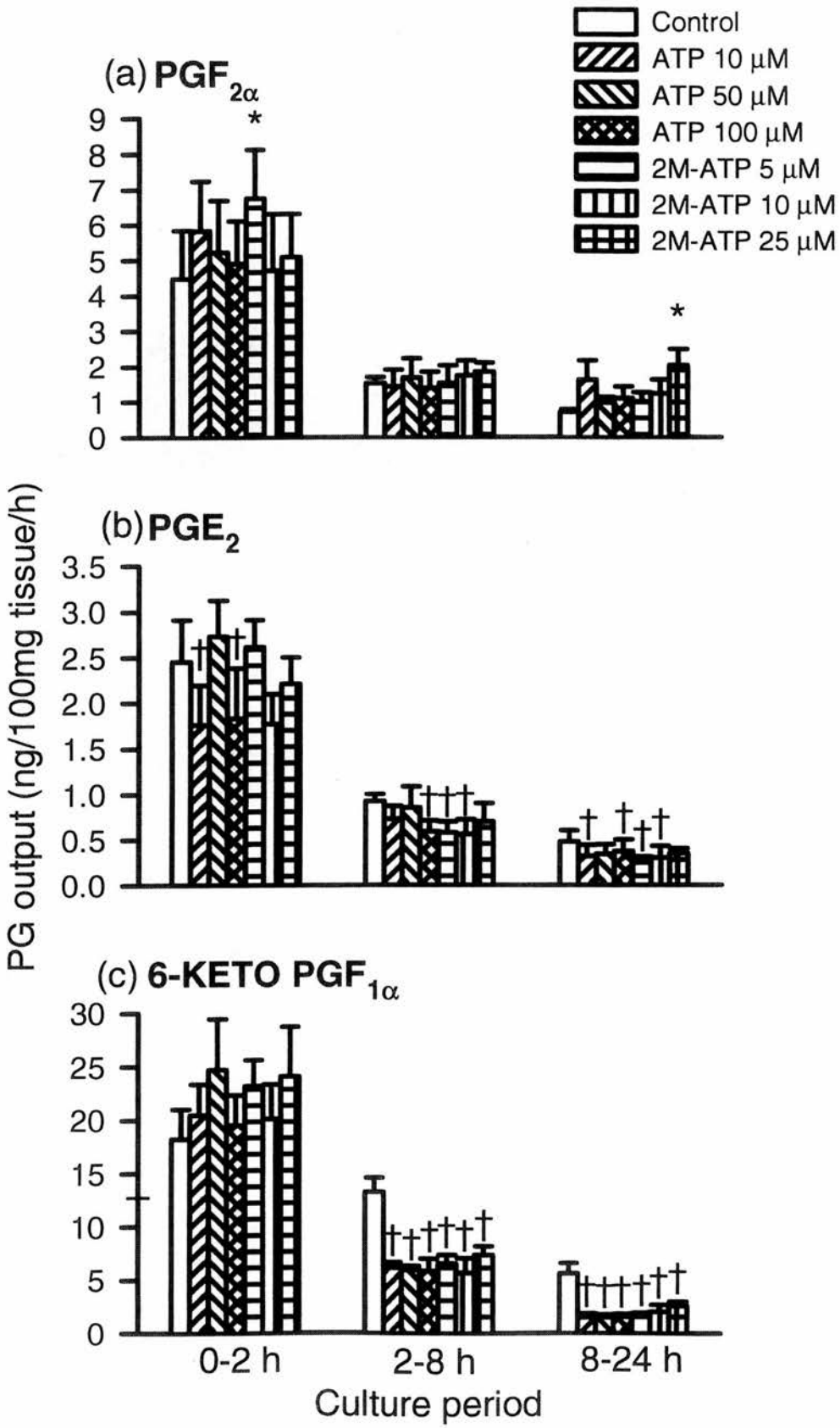


Figure 3A.2.2 Effects of ATP (10, 50 and 100 μm) and 2-methylthio-ATP (5, 10 and 25μm) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.
*/† Significantly higher/lower, $P < 0.05$, than corresponding control value.

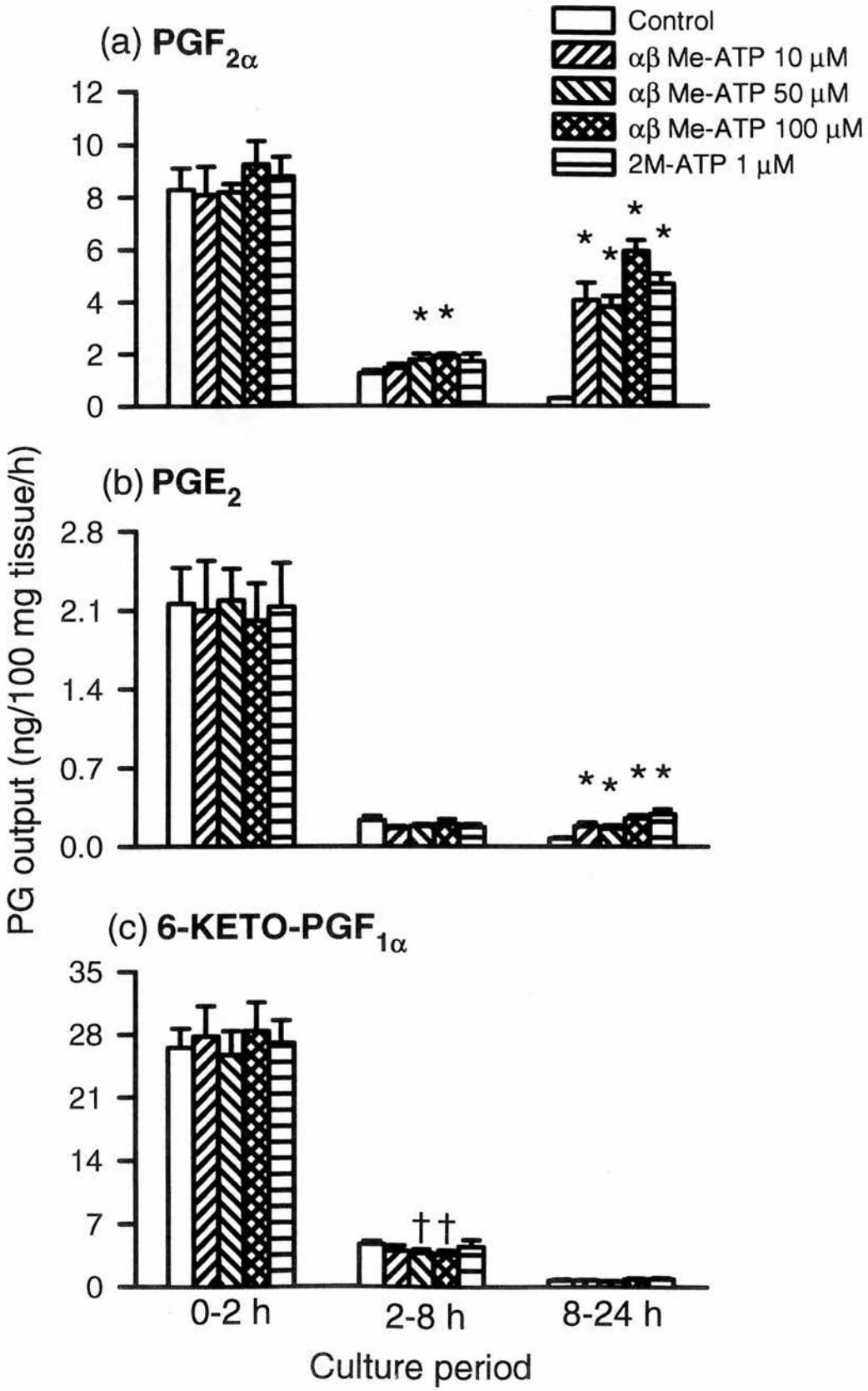


Figure 3A.2.3 Effects of αβ methylene-ATP (10, 50 and 100 μM) and 2 methylthio-ATP (1 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.

*/†Significantly higher/lower, $P < 0.05$, than corresponding control value.

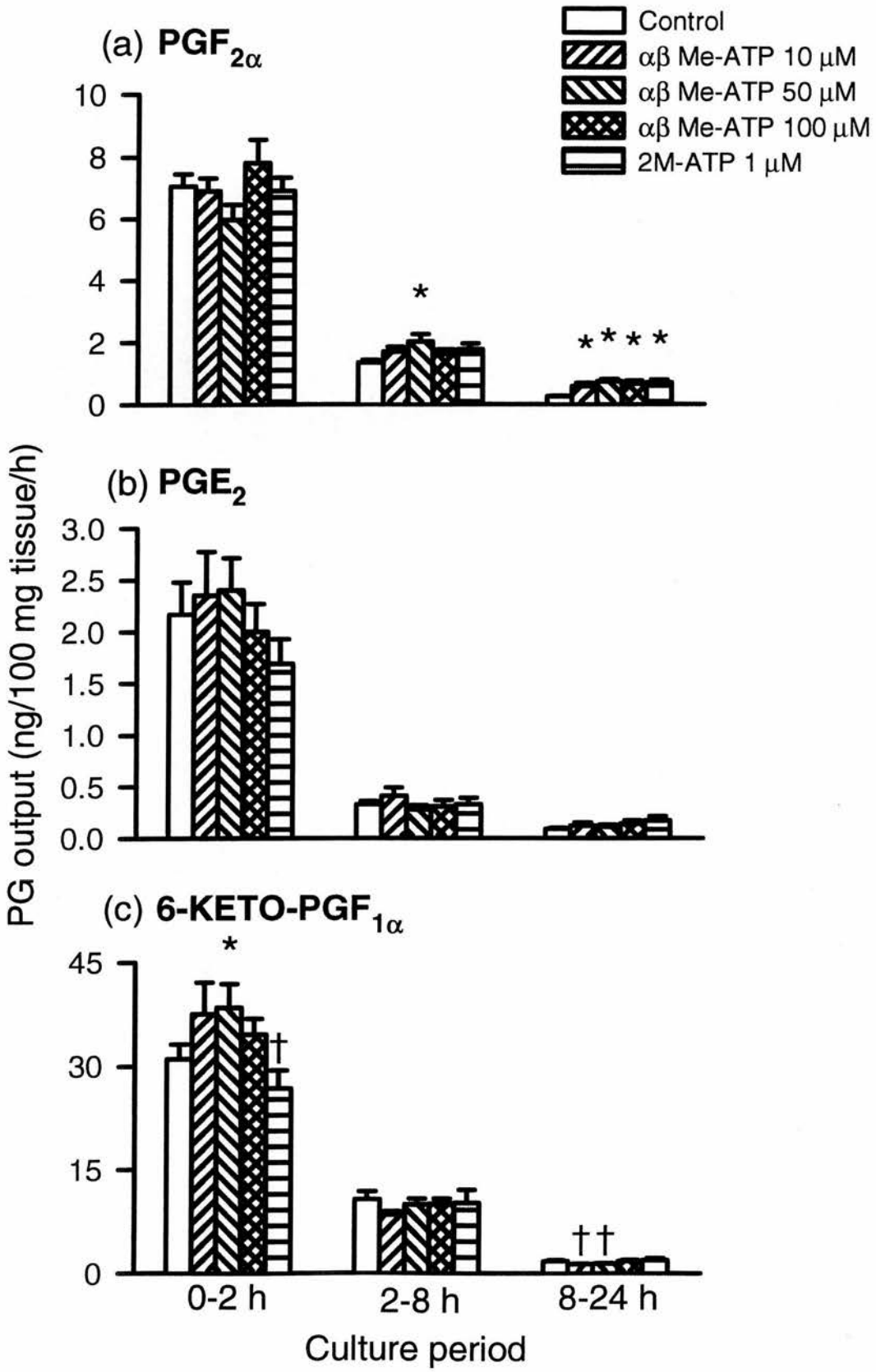


Figure 3A.2.4 Effects of αβ methylene-ATP (10, 50 and 100 μM) and 2 methylthio-ATP (1 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.

*/†Significantly higher/lower, $P < 0.05$, than corresponding control value.

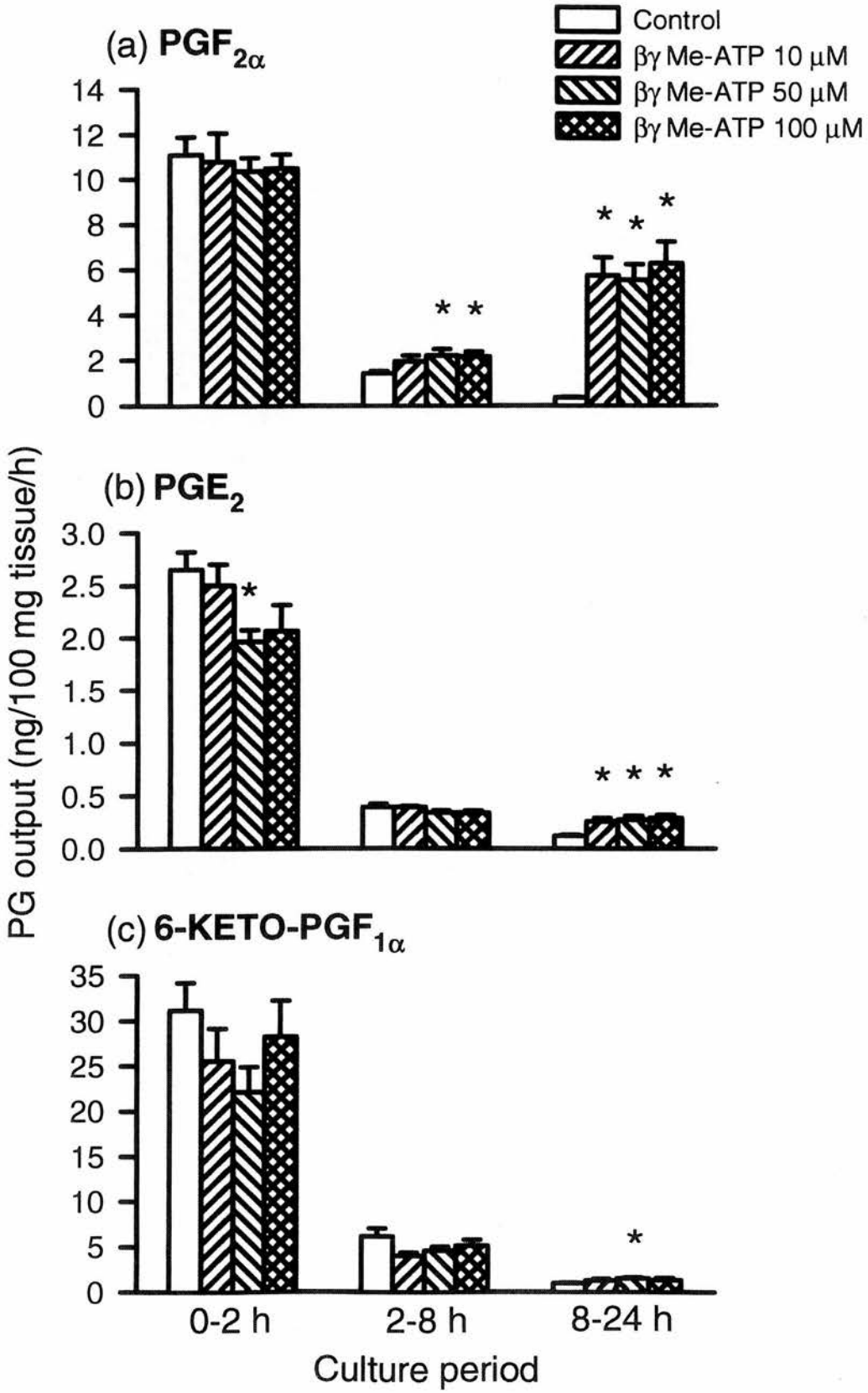


Figure 3A.2.5 Effect of βγ methylene-ATP (10, 50 and 100 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.
*Significantly higher, $P < 0.05$, than corresponding control value.

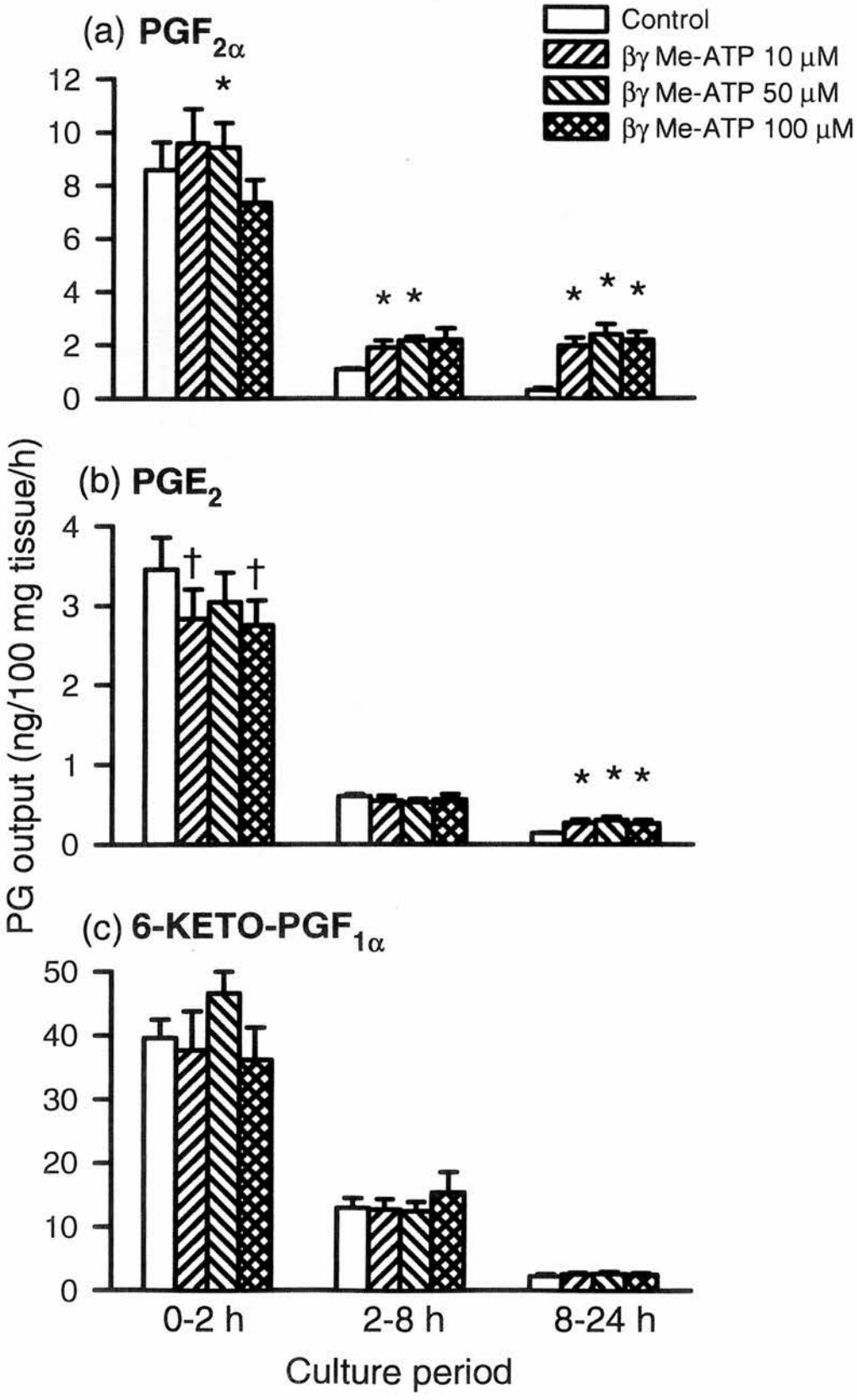


Figure 3A.2.6 Effect of βγ methylene-ATP (10, 50 and 100 μM) on mean (± SEM,n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.
*/†Significantly higher/lower, $P < 0.05$, than corresponding control value.

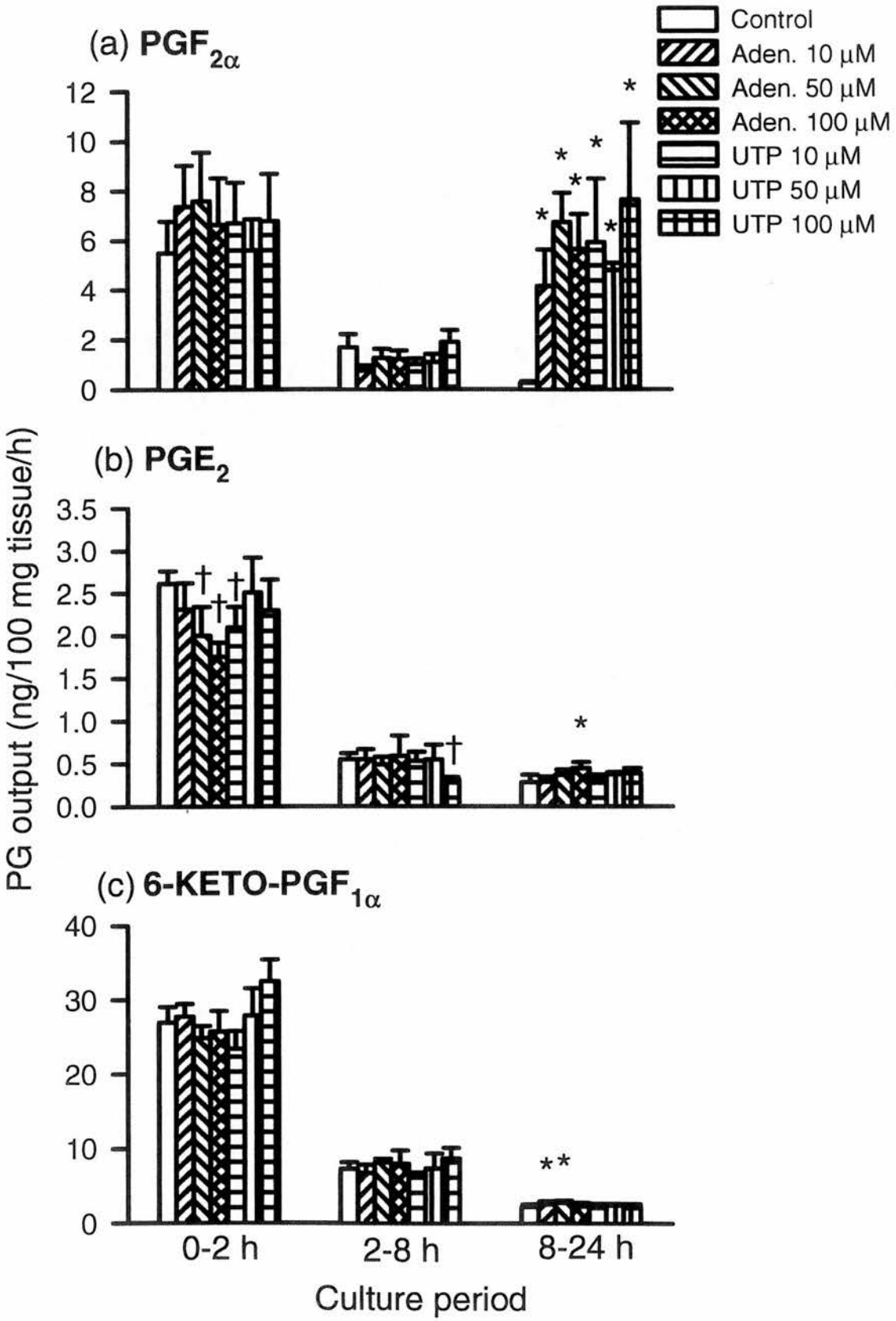


Figure 3A.2.7 Effects of adenosine (10, 50 and 100 μM) and UTP (10, 50 and 100 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.
*/†Significantly higher/lower, $P < 0.05$, than corresponding control value.

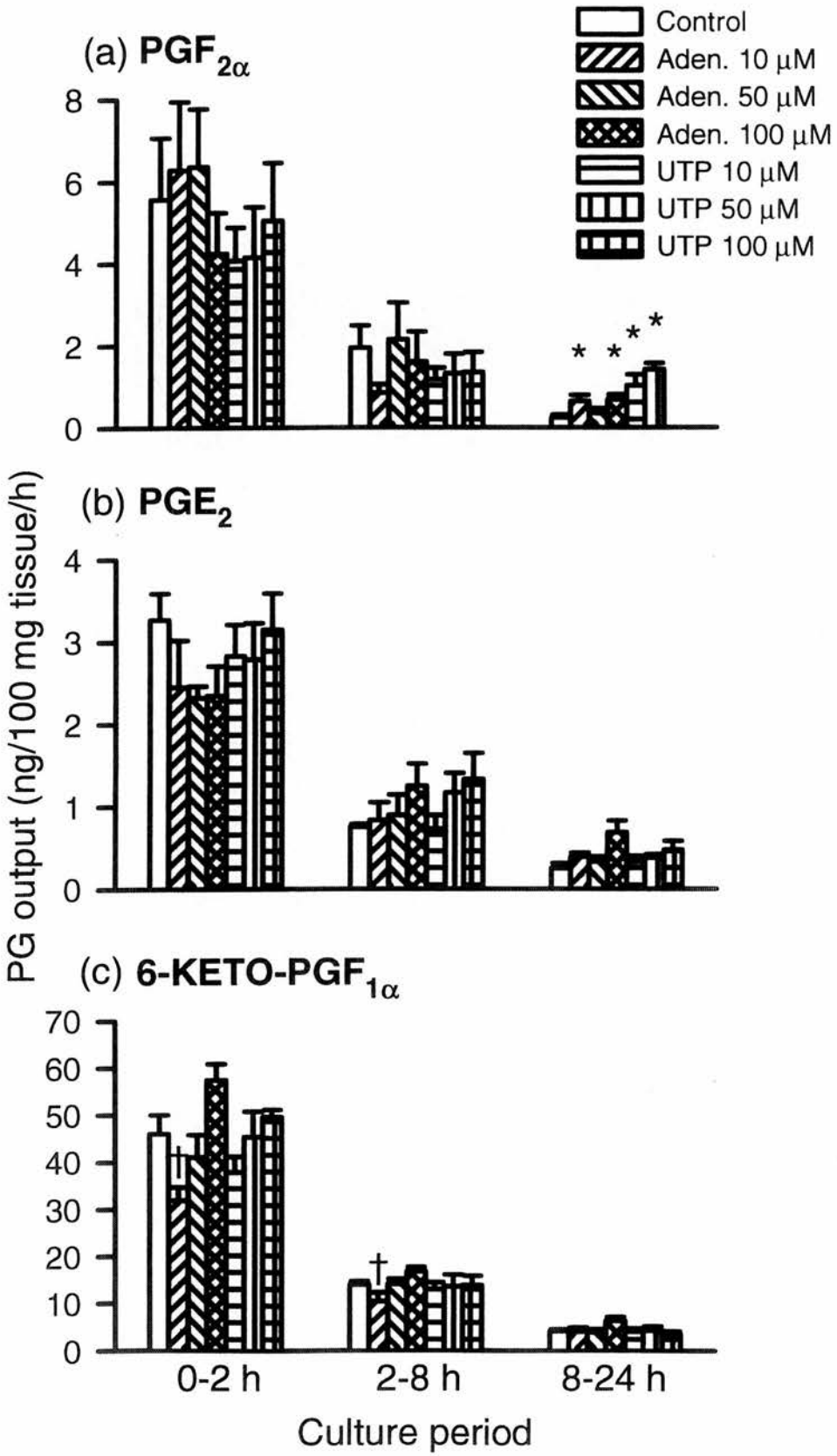


Figure 3A.2.8 Effects of adenosine (10, 50 and 100 μM) and UTP (10, 50 and 100 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.

*/†Significantly higher/lower, $P < 0.05$, than corresponding control value.

after 2 and 8 h of culture (Fig. 3A.2.8a). Adenosine (10, 50 and 100 mM) and UTP (10, 50 and 100 mM) had no effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ throughout the culture preriod, except adenosine (10 μM) which significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 2 and 8 h of culture (Fig. 3A.2.8c).

Experiment 3A.3 The Effects of Adenosine (A) Receptor and P2 Purinoceptor Antagonists on ATP- and Adenosine-Induced Increased Prostaglandin Output from Day 7 Guinea-Pig Uterus Superfused *In Vitro*.

Suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) alone had no effect on the outputs of any of the PGs measured (Figs. 3A.3.1-3A.3.4). ATP (100 μM) in the presence of suramin (100 μM) produced no significant increase in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus (Fig. 3A.3.1a). ATP (100 μM) significantly ($P < 0.05$, n=5) stimulated $PGF_{2\alpha}$ output in the presence of 8-sulphophenyltheophylline (140 μM) (Fig. 3A.3.2a). ATP (100 μM) in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 output from guinea-pig uterus (Figs. 3A.3.1b and 3A.3.2b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) increased by ATP (100 μM) in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Figs. 3A.3.1c and 3A.3.2c).

$PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) increased by adenosine (100 μM) in the presence of suramin (100 μM) (Fig. 3A.3.3a). Adenosine (100 μM) plus 8-

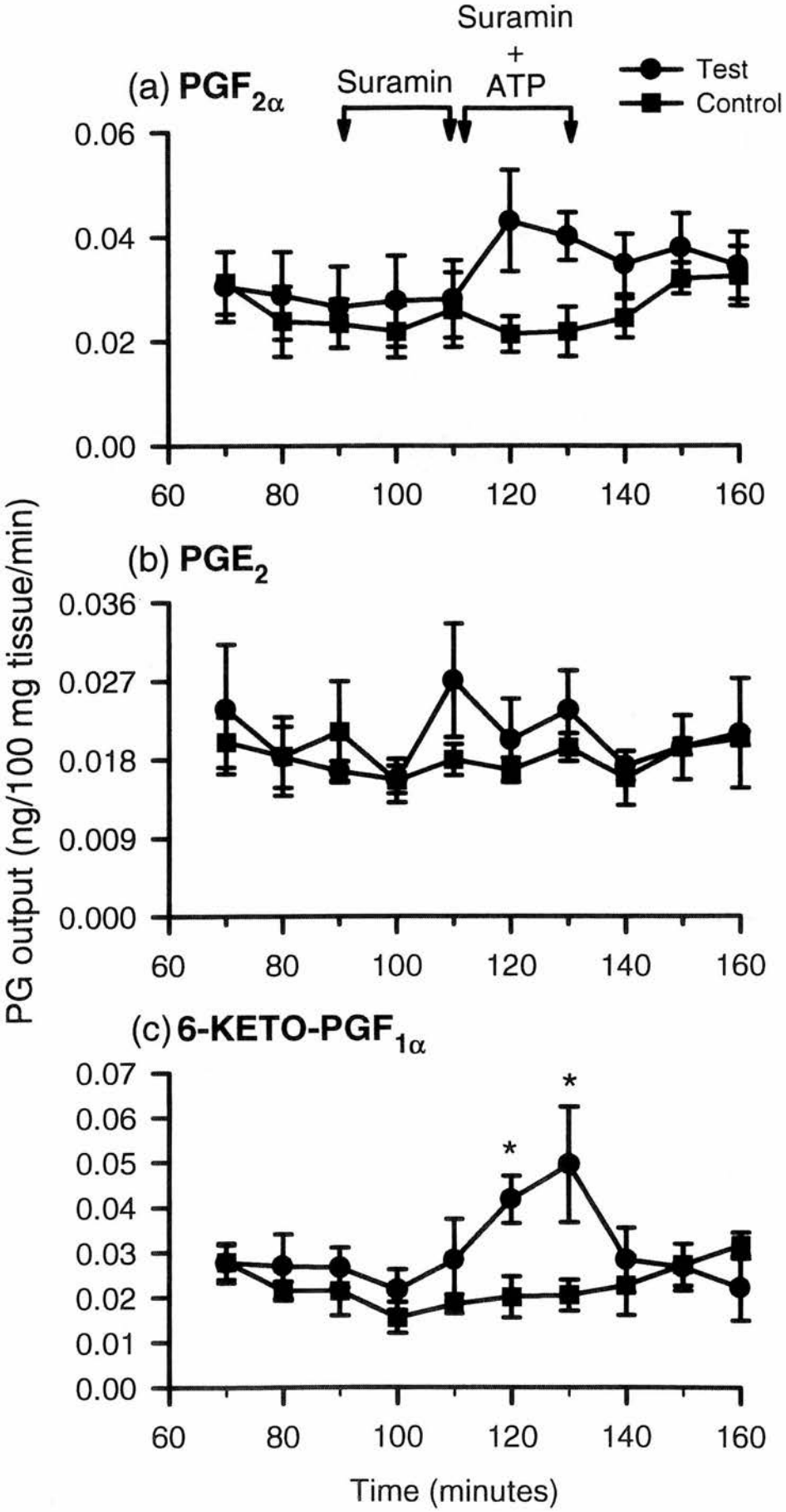


Figure 3A.3.1 Effects of suramin (100 μM) and ATP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.

*Significantly higher, $P < 0.05$, than value immediately preceding suramin plus ATP treatment.

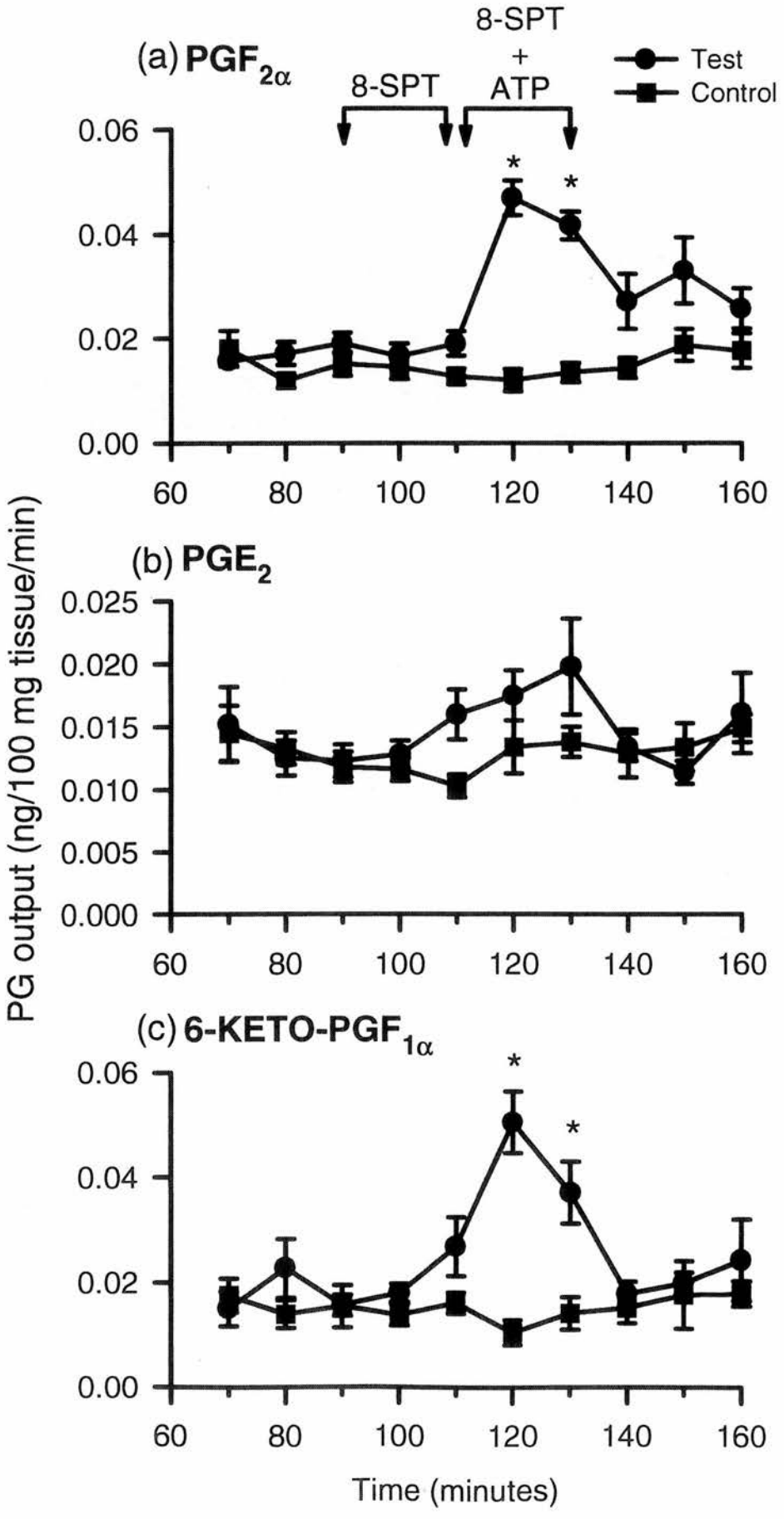


Figure 3A.3.2 Effects of 8-sulphophenyltheophylline (140 μM) and ATP (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.

*Significantly higher, $P < 0.05$, than value immediately preceding 8-sulphophenyltheophylline plus ATP treatment.

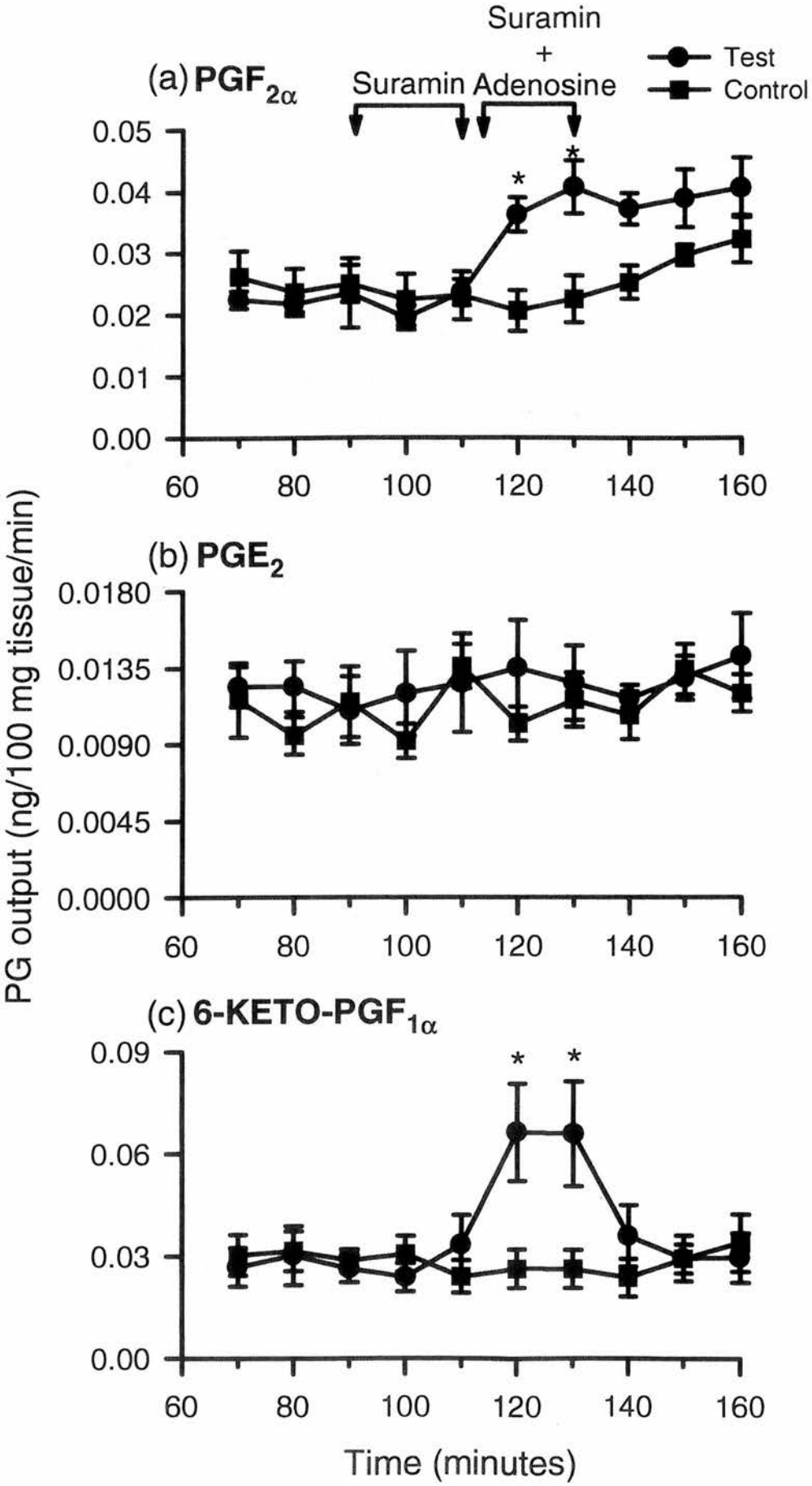


Figure 3A.3.3 Effects of suramin (100 μM) and adenosine (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding suramin plus adenosine treatment.

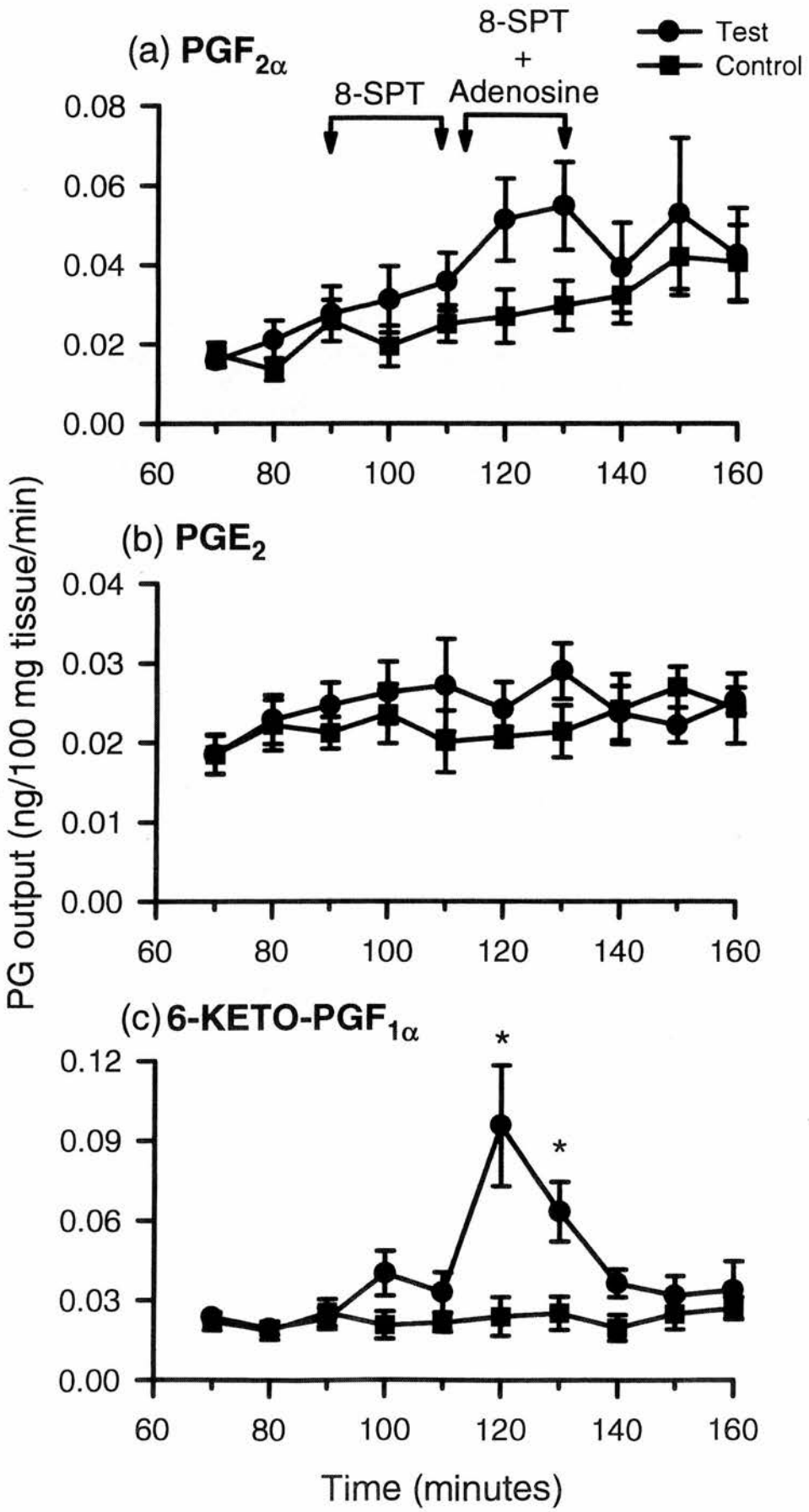


Figure 3A.3.4 Effects of 8-sulphophenyltheophylline (140 μM) and adenosine (100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 7 superfused guinea-pig uterus.
*Significantly higher, $P < 0.05$, than value immediately preceding 8-sulphophenyltheophylline plus adenosine treatment.

sulphophenyltheophylline (140 μM) had no significant effect on $PGF_{2\alpha}$ output (Fig. 3A.3.4a). Adenosine in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (100 μM) had no significant effect on PGE_2 output from superfused guinea-pig uterus (Figs. 3A.3.3b-3A.3.4b). 6-Keto-$PGF_{1\alpha}$ output was increased significantly ($P < 0.05$, n=5) by adenosine (100 μM) in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Figs. 3A.3.3c and 3A.3.4c).

Experiment 3A.4 The Effect of Adenosine (A) Receptor and P2 Purinoceptor Antagonists on ATP- and Adenosine-Induced Increased Prostaglandin Output from Day 7 Guinea-Pig Endometrium and Myometrium in Culture.

Endometrium

ATP (100 μM) alone significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output after 2 h and 24 h of culture (Fig. 3A.4.1a). Suramin (100 μM) had no effect on this ATP-induced increase in $PGF_{2\alpha}$ output after 2 h and 8-sulphophenyltheophylline had no effect on this ATP-induced increase in $PGF_{2\alpha}$ output after 2 h, and 24 h (Fig. 3A.4.1a). Suramin (100 μM) significantly ($P < 0.05$, n=5) decreased the ATP-induced increase in $PGF_{2\alpha}$ output from the guinea-pig endometrium after 24 h of culture although the output of $PGF_{2\alpha}$ in the presence of ATP and suramin was still significantly ($P < 0.05$, n=5) higher than the control value (Fig. 3A.4.1a). ATP (100 μM) plus suramin (100 μM) decreased $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5)

after 8 h when compared to the effect of ATP (100 μM) alone or no treatment, respectively (Fig. 3A.4.1a). PGE_2 output was significantly ($P < 0.05$, n=5) reduced by ATP (100 μM) plus suramin (100 μM) in the first 2 h of culture in comparison with the effect of ATP (100 μM) alone or no treatment. ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM) had no effect on PGE_2 output during the first 2 h of culture (Fig. 3A.4.1b). ATP (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) decreased PGE_2 output after 8 h and 24 h of culture (Fig. 3A.4.1b). After 8 and 24 h of culture, ATP (100 μM) plus suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 output when compared to PGE_2 output in the presence of ATP alone. ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM), but not ATP plus suramin (100 μM) or ATP (100 μM) alone significantly ($P < 0.05$, n=5) increased 6-keto-$PGF_{1\alpha}$ output during the first 2 h of culture (Fig. 3A.4.1c). ATP (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 8 h of culture (Fig. 3A.4.1c). Furthermore, 6-keto-$PGF_{1\alpha}$ output after 8 h of culture in the presence of ATP (100 μM) and suramin (100 μM), but not in the presence of ATP (100 μM) and 8-sulphophenyltheophylline (140 μM), was significantly ($P < 0.05$, n=5) lower than in the presence of ATP alone (Fig. 3A.4.1c). ATP (100 μM) alone and in the presence of suramin (100 μM), but not in the presence of 8-sulphophenyltheophylline (140 μM), significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 24 h of culture (Fig. 3A.4.1c). The presence of 8-sulphophenyltheophylline (140 μM) plus ATP (100 μM) had no significant effect on

$PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ outputs when compared to the effects of ATP (100 μM) alone (Fig. 3A.4.1a, b and c).

Adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output from guinea-pig endometrium after 2 h and 24 h of culture (Fig. 3A.4.3a). Adenosine (100 μM) plus suramin (100 μM) significantly ($P < 0.05$, n=5) reduced the adenosine-induced increase in $PGF_{2\alpha}$ output after 2 and 24 h (Fig. 3A.4.3a). After 8 h of culture, adenosine (100 μM) plus suramin (100 μM), but not plus 8-sulphophenyltheophylline (140 μM), significantly ($P < 0.05$, n=5) decreased $PGF_{2\alpha}$ output when compared to the no treatment control and to adenosine (100 μM) alone (Fig. 3A.4.3a). PGE_2 output was significantly ($P < 0.05$, n=5) reduced by adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) after 8 and 24 h, but not after 2 h, of culture when compared to the no treatment control (Fig. 3A.4.3b). Adenosine (100 μM) plus suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 output throughout the 24 h culture period when compared to the effect of adenosine (100 μM) alone (Fig. 3A.4.3b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) increased after 2 h of culture by adenosine (100 μM) plus 8-sulphophenyltheophylline (140 μM), but not by adenosine (100 μM) plus suramin (100 μM) or by adenosine (100 μM) alone (Fig. 3A.4.3c). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) reduced by adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 mM) after 8 h of culture (Fig. 3A.4.3c). After 24 h of culture, 6-keto-$PGF_{1\alpha}$ output was significantly (P

< 0.05, n=5) decreased by adenosine (100 μM) alone and in the presence of suramin (100 μM), but not in the presence of 8-sulphophenyltheophylline (140 μM) (Fig. 3A.4.3c). Furthermore, 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h of culture was significantly ($P < 0.05$, n=5) lower in the presence of adenosine (100 μM) plus suramin (100 μM) than in the presence of adenosine alone (Fig. 3A.4.3c).

Myometrium

ATP (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output after 2 h of culture (Fig.3A.4.2a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) decreased after 8 h by ATP (100 μM) plus suramin (100 μM), but not by ATP (100 μM) alone or in the presence of 8-sulphophenyltheophylline (140 μM), when compared to the no treatment controls (Fig.3A.4.2a). ATP (100 μM) plus suramin (100 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output after 8 h of culture when compared to the effect of ATP (100 μM) alone (Fig.3A.4.2a). ATP (100 μM) alone and plus suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on $PGF_{2\alpha}$ output after 24 h of culture (Fig.3A.4.2a). PGE_2 output was inhibited significantly ($P < 0.05$, n=5) by ATP (100 μM) alone, and in the presence of suramin (100 μM) throughout the 24 h culture period, and by ATP (100 μM) in the presence of 8-sulphophenyltheophylline (140 μM) after 8 h of culture but not after 2 and 24 h (Fig. 3A.4.2b). ATP in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 output when compared to the effect of ATP (100 μM) alone (Fig. 3A.4.2b). 6-Keto-$PGF_{1\alpha}$ output

was increased significantly ($P < 0.05$, n=5) in the first 2 h of culture and decreased significantly ($P < 0.05$, n=5) after 24 h by ATP (100 μM) alone, and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.4.2c). After 8 h of culture, 6-keto-$PGF_{1\alpha}$ output was reduced significantly ($P < 0.05$, n=5) by ATP (100 μM) plus suramin (100 μM), but not by ATP (100 μM) alone or in the presence of 8-sulphophenyltheophylline (140 μM) (Fig. 3A.4.2c). 6-Keto-$PGF_{1\alpha}$ output after 24 h, but not after 2 and 8 h, was significantly ($P < 0.05$, n=5) decreased further by ATP (100 μM) plus suramin (100 μM) but not plus 8-sulphophentltheophylline (140 μM) when compared to 6-keto-$PGF_{1\alpha}$ output in the presence of ATP (100 μM) alone (Fig. 3A.4.2c). ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM) had no significant effect on 6-keto-$PGF_{1\alpha}$ output when compared to ATP (100 μM) alone throughout the 24 h culture period (Fig. 3A.4.2c).

Adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) stimulated $PGF_{2\alpha}$ output in the first 2 h of culture (Fig.3A.4.4a). Adenosine (100 μM) alone but not in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) increased $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5) after 8 and 24 h of culture (Fig.3A.4.4a). Adenosine (100 μM) plus suramin (100 μM) significantly ($P < 0.05$, n=5) reduced this adenosine-induced increase in $PGF_{2\alpha}$ output after 8 and 24 h of culture (Fig.3A.4.4a). 8-Sulphophenyltheophylline (140 μM) had no significant effect on adenosine-induced increased $PGF_{2\alpha}$ output from guinea-pig myometrium (Fig. 3A.4.4a). PGE_2 output was significantly ($P < 0.05$, n=5) decreased by adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-

sulphophenyltheophylline (140 μM) after 8 h of culture (Fig.3A.4.4b). PGE_2 output was reduced significantly ($P < 0.05$, n=5) by adenosine (100 μM) plus suramin (100 μM), but not by adenosine plus 8-sulphophenyltheophylline (140 μM) or by adenosine (100 μM) alone, after 2 h and 24 h of culture (Fig.3A.4.4b). ATP in the presence of suramin (100 μM), or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 output when compared to the effect of ATP (100 μM) alone (Fig.3A.4.4b). 6-Keto-$PGF_{1\alpha}$ output was increased significantly ($P < 0.05$, n=5) by adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) after 2 h, but not after 8 and 24 h, of culture (Fig. 3A.4.4c). Adenosine (100 μM) alone had no significant effect on 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h of culture (Fig. 3A.4.4c). Adenosine (100 μM) plus suramin (100 μM) significantly ($P < 0.05$, n=5) decreased 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h of culture and adenosine (100 μM) plus 8-sulphophenyltheophylline (140 μM) significantly ($P < 0.05$, n=5) inhibited output after 24 h but not after 8 h, when compared to untreated controls (Fig. 3A.4.4c). Adenosine (100 μM) plus suramin (100 μM) significantly ($P < 0.05$, n=5) decreased 6-keto-$PGF_{1\alpha}$ output after 8 h and 24 h when compared to the effect of adenosine (100 μM) alone (Fig. 3A.4.4c). Adenosine (100 μM) plus 8-sulphophenyltheophylline (140 μM) reduced 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 24 h, but not after 2 and 8 h, when compared to the effect of adenosine (100 μM) alone (Fig. 3A.4.4c).

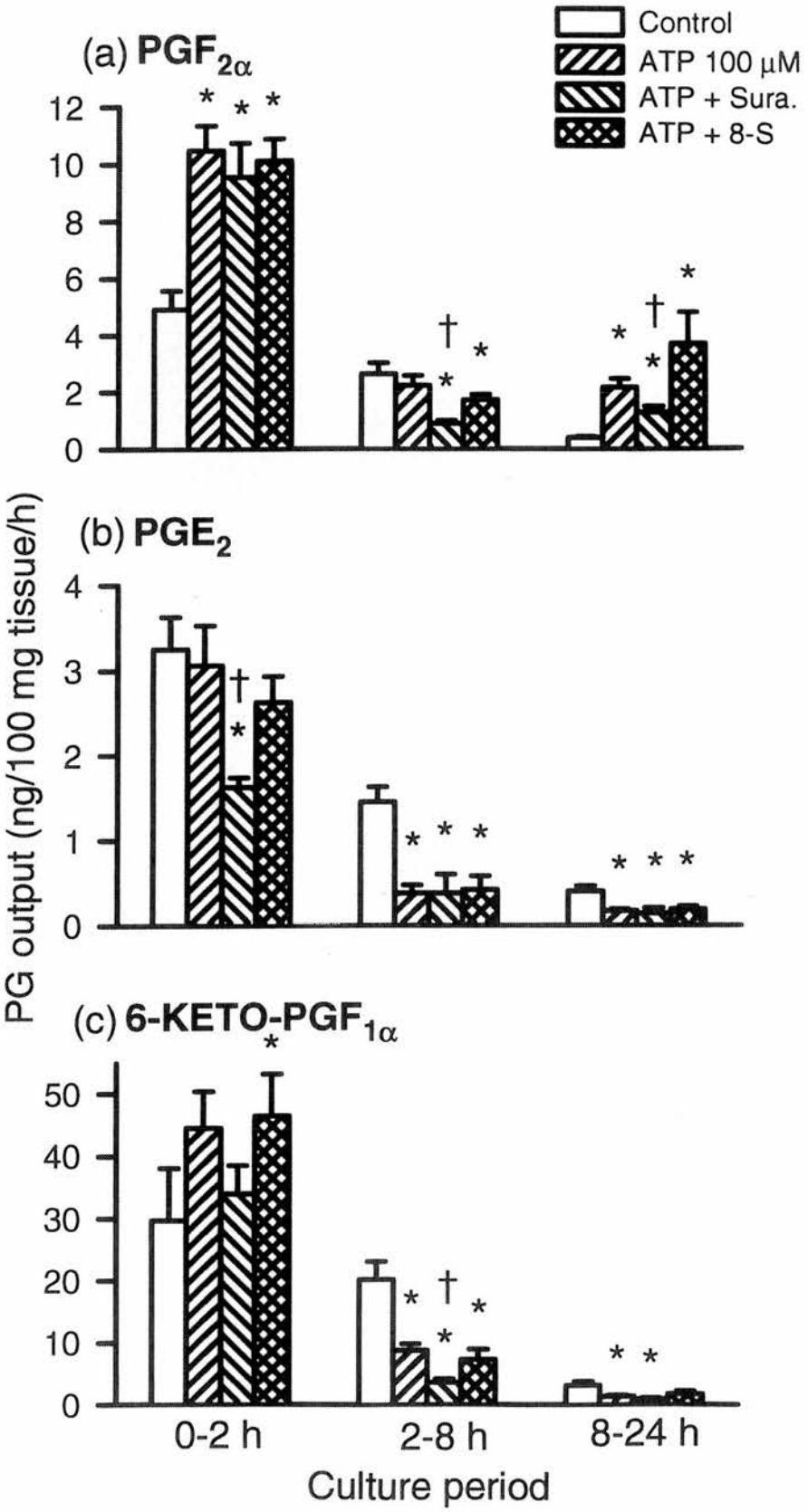


Figure 3A.4.1 Effects of ATP (100 μM) plus suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.
*Significantly different, $P < 0.05$, than corresponding control value.
†Significantly lower, $P < 0.05$, than corresponding ATP value.

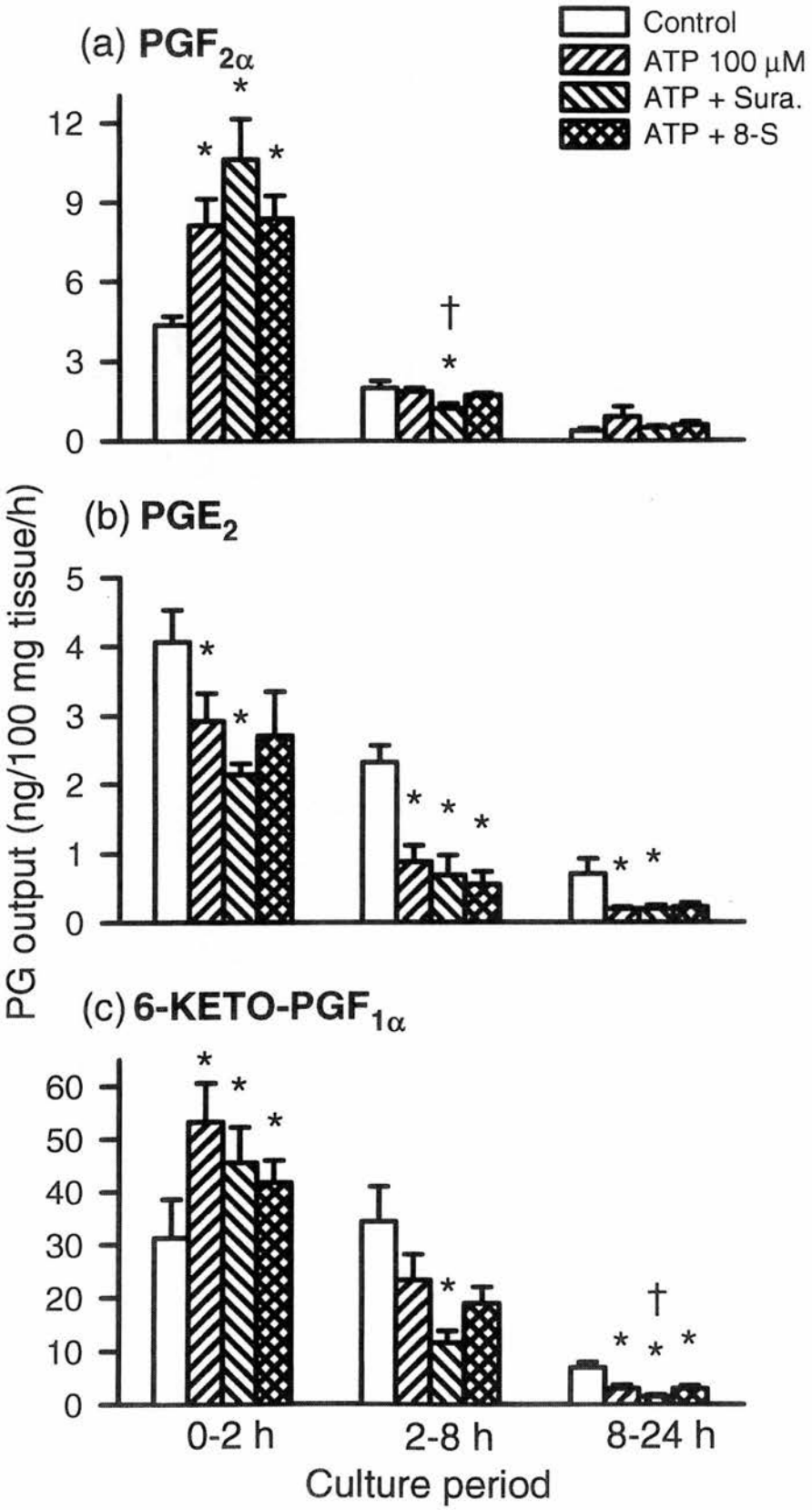


Figure 3A.4.2 Effects of ATP (100 μM) plus suramin (100 mM) or 8-sulphophenyltheophylline (140 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.

*Significantly different, $P < 0.05$, than corresponding control value.
†Significantly lower, $P < 0.05$, than corresponding ATP value.

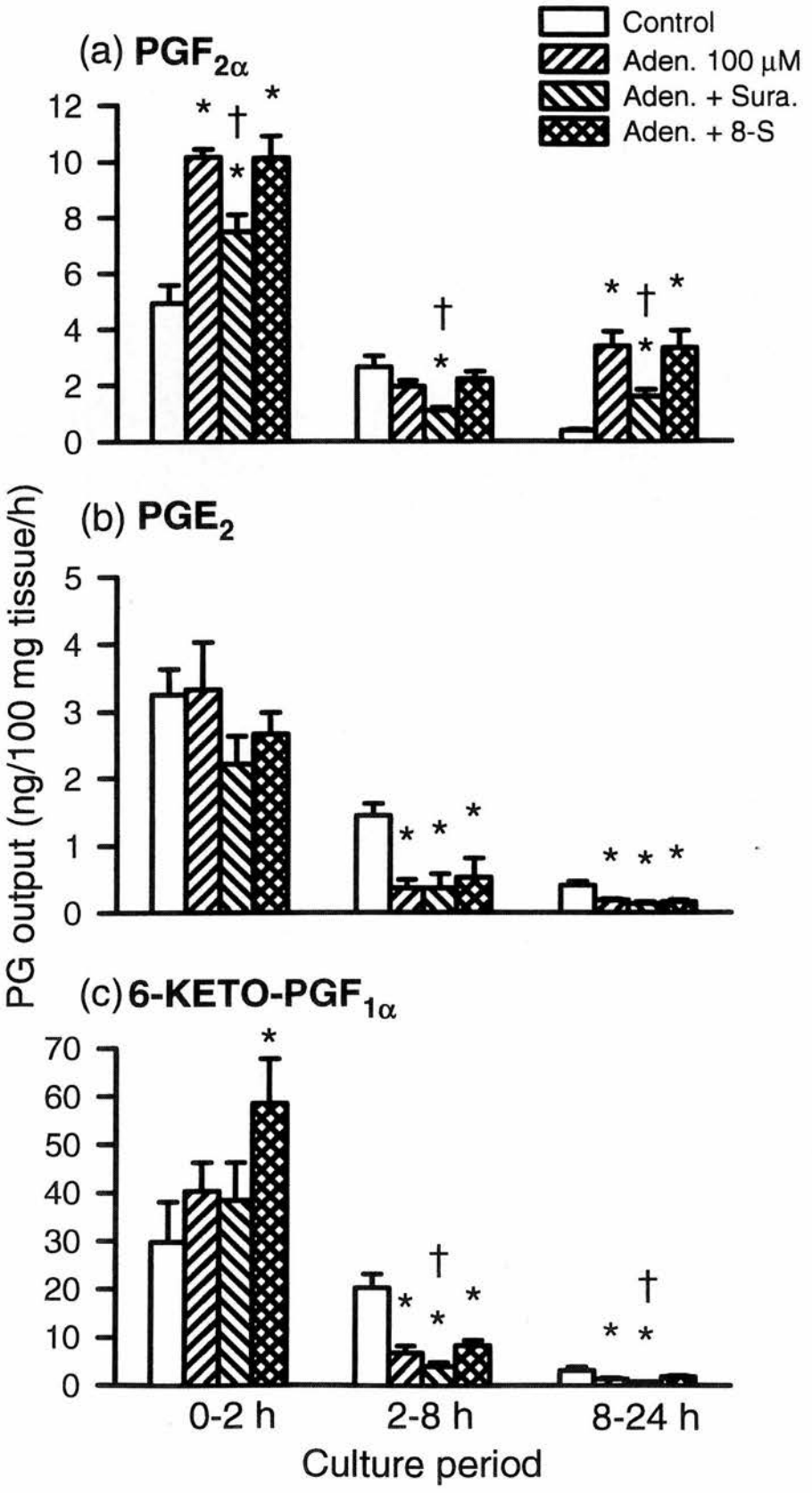


Figure 3A.4.3 Effects of adenosine (100 μM) plus suramin (100 mM) or 8-sulphophenyltheophylline (140 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig endometrium cultured for 24 h.
*Significantly different, $P < 0.05$, than corresponding control value.
†Significantly lower, $P < 0.05$, than corresponding adenosine value.

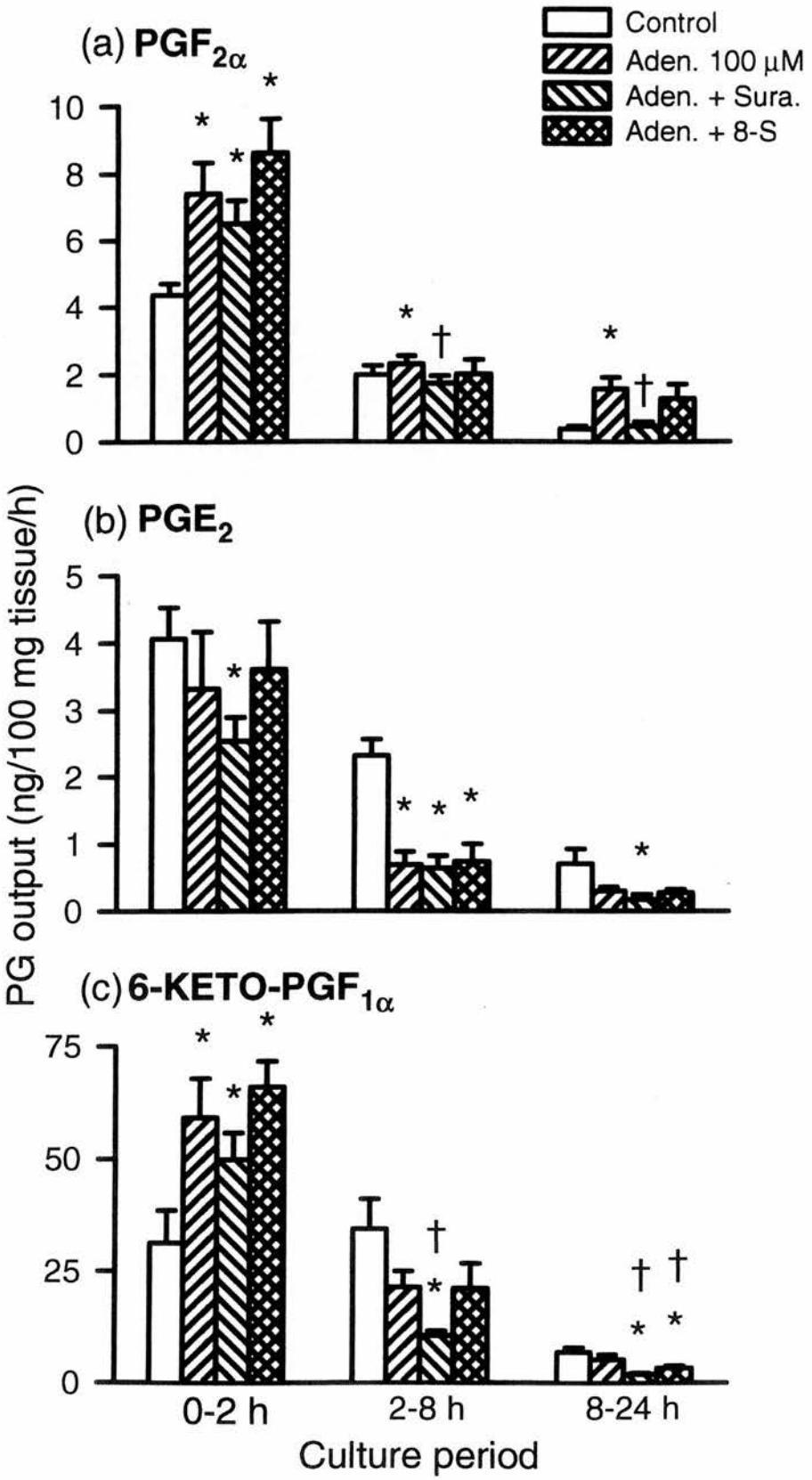


Figure 3A.4.4 Effects of adenosine (100 μM) plus suramin (100 mM) or 8-sulphphenyltheophylline (140 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from guinea-pig myometrium cultured for 24 h.

*Significantly different, $P < 0.05$, than corresponding control value.
†Significantly lower, $P < 0.05$, than corresponding adenosine value.

Experiment 3A.5 Prostaglandin Production by Homogenates of Day 7 Guinea-Pig Endometrium and Myometrium after 24 h of Culture.

Endometrium

$PGF_{2\alpha}$ production by homogenates of endometrium was significantly ($P < 0.05$, n=4) increased following 24 h culture of the endometrium with ATP (100 μM) or adenosine (100 μM) alone, and by ATP (100 μM) and adenosine (100 μM) in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.1a). There were no significant differences between $PGF_{2\alpha}$ production following treatment with ATP (100 μM) or adenosine (100 μM) alone when compared to $PGF_{2\alpha}$ production with ATP or adenosine in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.1a). ATP (100 μM) and adenosine (100 μM) on their own or in the presence of suramin (100 μM) had no significant effect on PGE_2 production (Fig. 3A.51b). PGE_2 production was significantly ($P < 0.05$, n=4) increased following 24 h culture in the presence of ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM) and adenosine (100 μM) plus 8-sulphophenyltheophylline (140 μM) when compared to the untreated control (Fig. 3A.5.1b). ATP in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) had no significant effect on PGE_2 production when compared to the effect of ATP (100 μM) or adenosine (100 μM) alone (Fig. 3A.5.1b). None of the treatments used had any significant effect on 6-keto-$PGF_{1\alpha}$ production by endometrial homogenates (Fig. 3A.5.1c).

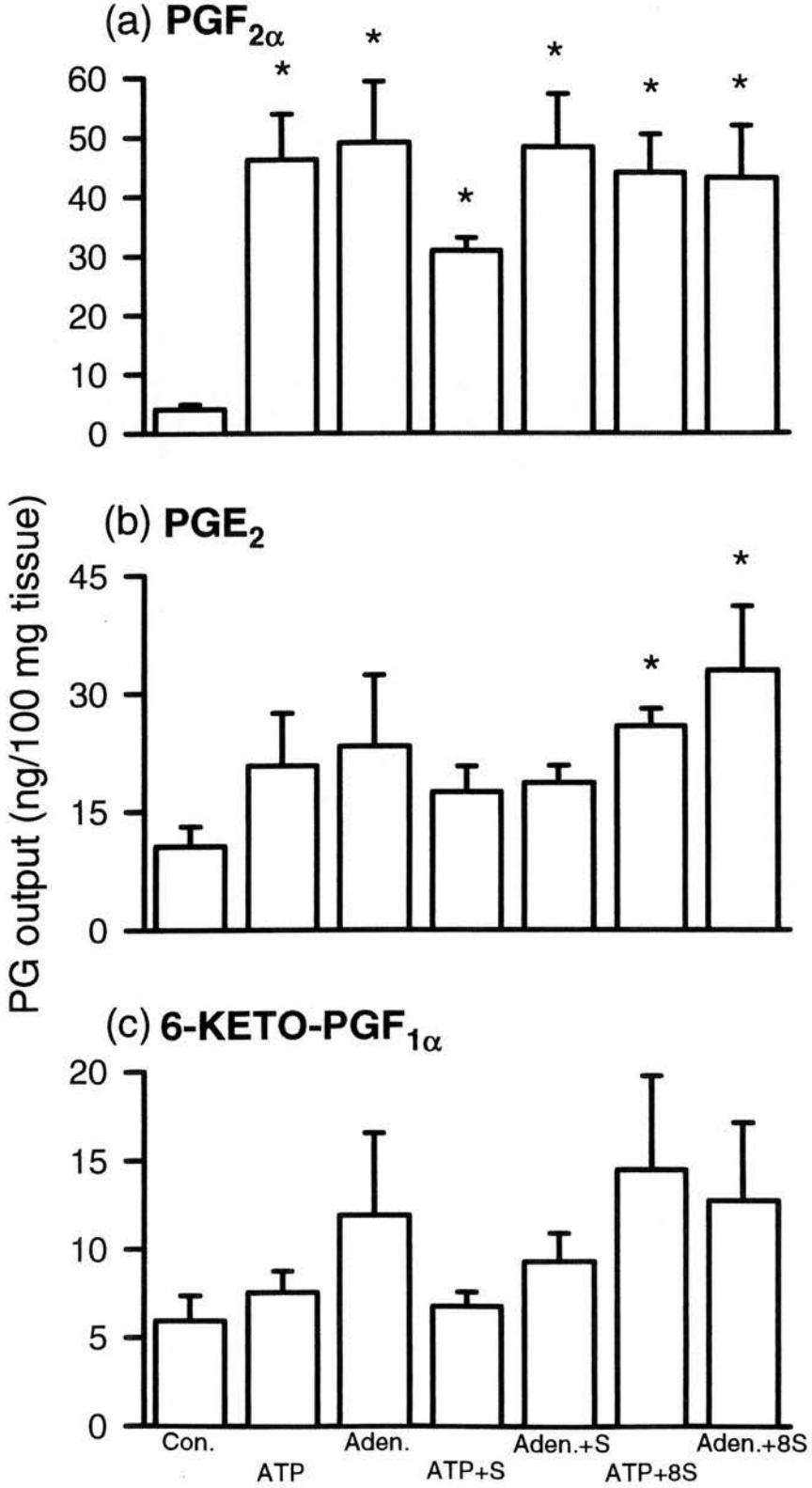


Figure 3A.5.1 Production of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ by endometrial homogenates after 24 h in culture with ATP (100 μM) and adenosine (100 μM) alone and plus either suramin (100 μM) or 8-sulphophenyltheophylline (140 μM).
*Significantly higher, $P < 0.05$, than corresponding control value.

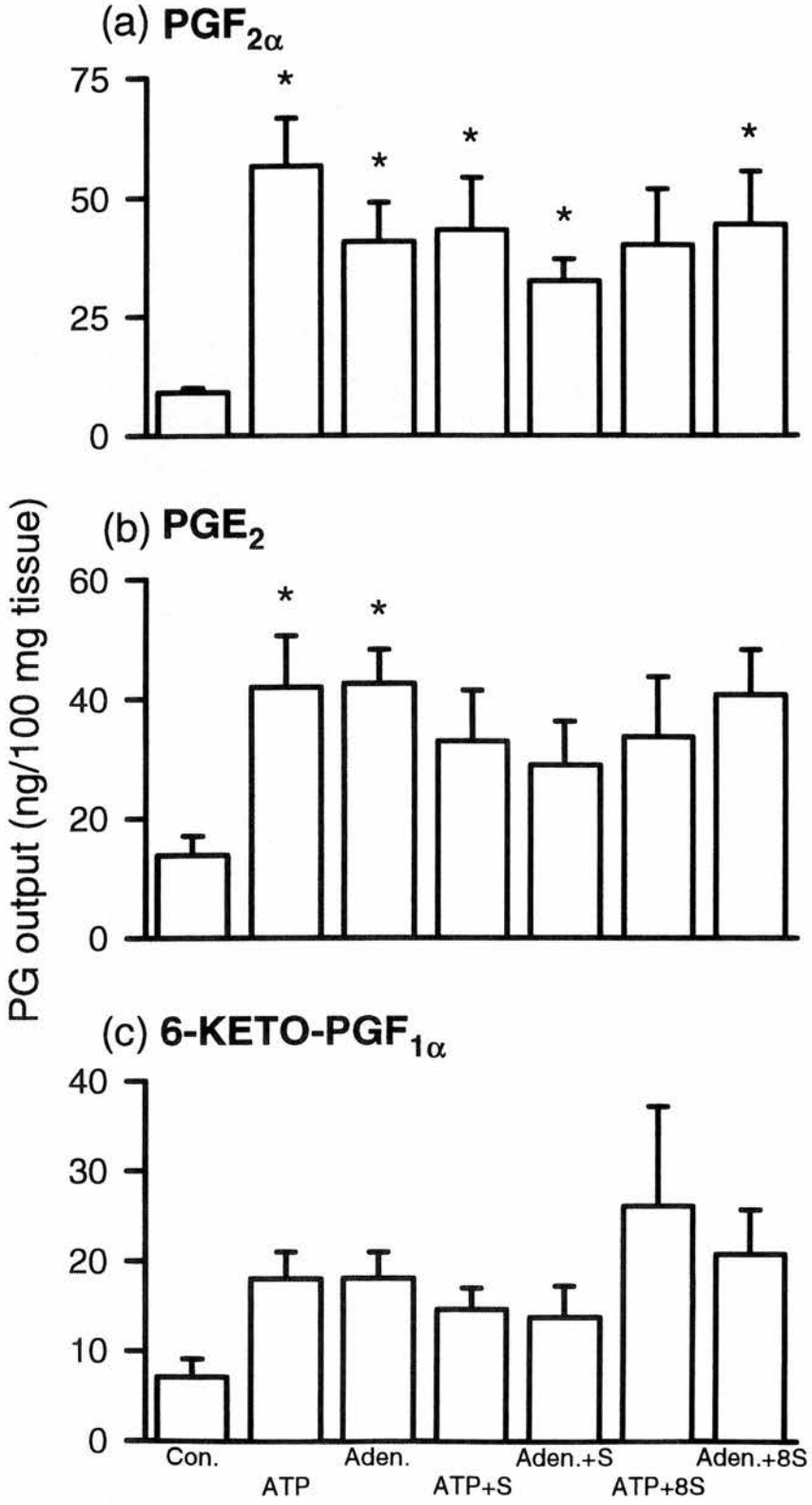


Figure 3A.5.2 Production of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ by myometrial homogenates after 24 h in culture with ATP (100 μM) and adenosine (100 μM) alone and plus either suramin (100 μM) or 8-sulpho-phenyltheophylline (140 μM).
*Significantly higher, $P < 0.05$, than corresponding control value.

Myometrium

$PGF_{2\alpha}$ production by homogenates of the myometrium was significantly ($P < 0.05$, n=4) increased following 24 h culture with ATP (100 μM) and adenosine (100 μM) alone and in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM), except with ATP (100 μM) plus 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.2a). There were no significant differences between $PGF_{2\alpha}$ production following treatment with ATP (100 μM) or adenosine (100 μM) alone when compared to $PGF_{2\alpha}$ production with ATP or adenosine in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.2a). PGE_2 production by homogenates was increased significantly ($P < 0.05$, n=4) following treatment with ATP (100 μM) and adenosine (100 μM) alone, but not by ATP and adenosine in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.2b). There were no significant differences between PGE_2 production following ATP (100 μM) or adenosine (100 μM) treatment alone when compared to treatment with ATP and adenosine in the presence of suramin (100 μM) or 8-sulphophenyltheophylline (140 μM) (Fig. 3A.5.2b). 6-Keto-$PGF_{1\alpha}$ output was not significantly affected by any of the treatments used (Fig. 3A.5.2c).

Discussion:

This study has shown that ATP, 2 methylthio-ATP and adenosine stimulate $PGF_{2\alpha}$ and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) outputs from the non-pregnant guinea-pig uterus superfused *in vitro*. $PGF_{2\alpha}$ output was also stimulated by UTP while 6-keto-$PGF_{1\alpha}$ output was not. αβ Methylene-ATP and βγ methylene-ATP had no

significant effect on the outputs of either $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ at the concentrations used. The ATP analogues that did not stimulate $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs may be less potent than ATP and may therefore require even higher concentrations than those used to stimulate $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs significantly. Piper & Hollingsworth (1996) observed that βγ methylene-ATP was less effective at contracting the guinea-pig uterus when compared to the effects of ATP and other ATP analogues. ATP, analogues of ATP, adenosine and UTP had no effect on PGE_2 output from the superfused guinea-pig uterus.

Both adenosine and ATP stimulated $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs suggesting the presence of A and P2 receptors in the guinea-pig uterus. However, stimulation of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs by ATP is not enough to prove that P2 receptors are present in the guinea-pig uterus as ATP is rapidly metabolised to adenosine, which acts at A receptors. Studies with P2 and A receptor antagonists, suramin and 8-sulphophenyltheophylline, respectively, were performed to determine whether ATP or adenosine increased $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the superfused uterus by acting on P2 purinoceptors or A receptors, respectively. Suramin or 8-sulphophenyltheophylline treatment alone had no effect on $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ outputs from the superfused uterus. Suramin inhibited the stimulatory effect of ATP on $PGF_{2\alpha}$ output from the superfused guinea-pig uterus while 8-sulphophenyltheophylline had no such effect. Therefore, this indicates that ATP acts via P2 purinoceptors and not on A receptors, even though ATP may be broken down rapidly by ecto-nucleotidases to adenosine. The stimulatory effect of adenosine on $PGF_{2\alpha}$ output from superfused uterus was inhibited by 8-sulphophenyltheophylline

while suramin had no effect on adenosine stimulated $PGF_{2\alpha}$ output which suggests that A receptors are also present in guinea-pig uterus and mediate the effect of adenosine. These results are consistent with the work of Smith *et al.* (1988) who observed that both A and P2 receptors were present in the guinea-pig myometrium. The stimulation of 6-keto-$PGF_{1\alpha}$ output by ATP and adenosine from superfused uterus was not affected by the presence of suramin or 8-sulphophenyltheophylline. Therefore, ATP- and adenosine-induced increases in 6-keto-$PGF_{1\alpha}$ output appear to be via a different mechanism than their stimulation of $PGF_{2\alpha}$ output from superfused guinea-pig uterus. The stimulatory effects of ATP and adenosine on 6-keto-$PGF_{1\alpha}$ output from the superfused uterus may be receptor mediated but may require high concentrations of antagonist to block the 6-keto-$PGF_{1\alpha}$ pathway compared to the concentration required to block the $PGF_{2\alpha}$ pathway. Alternatively, stimulation of 6-keto-$PGF_{1\alpha}$ output from the superfused guinea-pig uterus may be non-receptor mediated and this might explain why suramin and 8-sulphophenyltheophylline, P2 and A receptor anatagonists respectively, have no effect on ATP and adenosine stimulated 6-keto-$PGF_{1\alpha}$ output. ATP could be stimulating 6-keto-$PGF_{1\alpha}$ output from the superfused uterus by phosphorylating PLA_2. PLA_2 is the enzyme responsible for arachidonic acid release from membrane phospholipids. However, this does not account for the stimulatory action of adenosine. The mechanism for 6-keto-$PGF_{1\alpha}$ production by the superfused guinea-pig uterus remains unclear and requires further investigation.

None of the treatments used had any effect on PGE_2 output suggesting a different mechanism for PGE_2 production by the guinea-pig uterus in the short-term

superfusion experiments from the mechanisms for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production. There is evidence that different pools of arachidonic acid exist in tissues and that these pools are specifically channelled to different PGHS enzymes (Reddy & Herschman, 1994). These pools of arachidonic acid could also be channelled to specific types of PG synthesis depending on which PG is required. Naraba *et al.* (1998) demonstrated that the constitutive enzyme, PGHS-1, and the inducible enzyme, PGHS-2, are preferentially coupled to distinct prostanoid synthases in rat peritoneal macrophages. This could also be the case in guinea-pig uterus.

The increased PG outputs from superfused guinea-pig uterus observed in the presence of adenosine, ATP and ATP analogues is probably due to an increase in arachidonic acid release from the *sn*-2 position of membrane phospholipids. The release of arachidonic acid is attributed to the enzyme, phospholipase A_2 (PLA_2) (Flower & Blackwell, 1976). This is the rate-limiting step in PG production (Vogt, 1978). Addition of exogenous arachidonic acid and PLA_2 have been observed to stimulate PG synthesis from the superfused guinea-pig uterus (Poyser, 1987a). Previous work has also shown that melittin, a PLA_2 activator, stimulates PG output from the guinea-pig uterus (Johnson & Poyser, 1991), while aristolochic acid, a PLA_2 inhibitor, reduces PG output from cultured cells from the guinea-pig endometrium (Naderali & Poyser, 1996b). At least three phospholipase A_2 enzymes have been implicated in the mobilisation of arachidonic acid from phospholipids (Balsinde & Dennis, 1996). However, cytosolic PLA_2 ($cPLA_2$) is selective for the hydrolysis of phospholipid containing arachidonic acid (Clark *et al.*, 1991; Kramer *et al.*, 1991) and can be quickly activated by post-translational mechanisms to rapidly mobilise arachidonic acid for eicosanoid production (Leslie, 1997). It remains to be

seen which of the PLA_2 enzymes are present in the guinea-pig uterus and which are involved in PG production. However, it seems likely that ATP, ATP analogues, UTP and adenosine stimulate PG output from the superfused guinea-pig uterus by activating one or more types of PLA_2.

After 24 h of culture ATP, αβ methylene-ATP, 2 methylthio-ATP, βγ methylene-ATP, UTP and adenosine stimulated $PGF_{2\alpha}$ output from guinea-pig uterus, particularly from the endometrium. As very little stimulation was observed after 2 and 8 h of culture, it appears that the mechanism for increased $PGF_{2\alpha}$ production after 24 h of culture is different than the one involved in increased $PGF_{2\alpha}$ production by guinea-pig uterus in the short-term superfusion experiments. While it appears likely that increased mobilisation of arachidonic acid is the reason for increased PG production from the superfused uterus, increased PG production from the cultured uterus, particularly the endometrium after 24 h of culture, is probably due to stimulation of PGHS synthesis, the enzyme responsible for PG synthesis from arachidonic acid (Lands, 1979). Homogenisation of endometrium and myometrium showed an increase in $PGF_{2\alpha}$ production following 24 h treatment with adenosine and ATP. These homogenisation experiments are an indication of PG synthesising capacity and therefore the amount of PGHS present in the tissue before homogenisation. These results suggest that ATP and adenosine stimulate PGHS synthesis, the conversion of arachidonic acid to PGH_2 and an increase in $PGF_{2\alpha}$ production from the guinea-pig endometrium and myometrium after 24 h culture. This may not be the mechanism for increased $PGF_{2\alpha}$ output in short-term superfusion

experiments as it is unlikely that any increase in PGHS synthesis would be observed in such a short period of time. A decrease in $PGF_{2\alpha}$ metabolism may also be the cause of the increase in $PGF_{2\alpha}$ output observed after 24 h of culture. Levels of 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), the metabolite of $PGF_{2\alpha}$, were measured in the control samples of culture medium following 24 h of culture, by ELISA assay and were found to be undetectable in control tissues, suggesting that metabolism of $PGF_{2\alpha}$ after 24 h of culture was low. This observation is consistent with metabolism by guinea-pig uterus homogenates being low (< 5%; Poyser, 1979). Therefore, these results suggest that increased $PGF_{2\alpha}$ production by the guinea-pig uterus following 24 h of culture with ATP and adenosine is due to increased synthesis, probably through the increased production of PGHS. ATP or adenosine-induced increases in $PGF_{2\alpha}$ production by homogenates of the guinea-pig uterus (endometrium and myometrium) were not inhibited in the presence of either suramin or 8-sulphophenyltheophylline after 24 h of culture. This suggests that ATP- and adenosine-induced increase in PGHS synthesis may not have been mediated by receptor activation at P2 and A receptors, respectively.

The effects of adenosine, ATP and ATP analogue treatment on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from guinea-pig uterus after 24 h culture were variable. Short-term superfusion of the guinea-pig uterus in the presence of adenosine, ATP and ATP analogues showed no stimulation of PGE_2 output. However, following 24 h of culture, PGE_2 output from the guinea-pig uterus, particularly from the endometrium, was significantly increased by $\alpha\beta$ methylene-ATP and $\beta\gamma$ methylene-ATP. These compounds were ineffective at stimulating PG outputs from superfused guinea-pig

uterus. Following 24 h of culture, an increase in PGHS synthesis may be directed towards PGE_2 production as well as $PGF_{2\alpha}$ production. Homogenates of endometrium and myometrium following 24 h of culture in the presence of adenosine and ATP, with or without suramin or 8-sulphophenyltheophylline, showed an increase in PGE_2 production, although this was not always significantly. This indicates increased capacity to synthesise PGE_2 and therefore increased PGHS present in the tissue before homogenisation. Therefore, it appears likely that increased PGE_2 output after 24 h of culture in the presence of αβ methylene-ATP and βγ methylene-ATP is a result of increased PGHS synthesis. PGE_2 output from the myometrium was inhibited by ATP and 2 methylthio-ATP throughout culture, and 6-keto-$PGF_{1\alpha}$ output from the endometrium and myometrium was inhibited in the long-term culture experiments in the presence of ATP, and 2 methylthio-ATP and, to some extent by αβ methylene-ATP. Homogenates of endometrium and myometrium following 24 h culture with ATP or adenosine alone or with either suramin or 8-sulphophenyltheophylline showed no significant increase in 6-keto-$PGF_{1\alpha}$ production following 24 h incubation. This suggests that the synthesising capacity for 6-keto-$PGF_{1\alpha}$ production was not increased following 24 h culture. It appears that most of the increased PGH_2 produced by the increase in PGHS is directed into the $PGF_{2\alpha}$-forming pathway and this channelling of PGH_2 towards $PGF_{2\alpha}$ formation may explain why PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs decreased after 24 h of culture.

Culture experiments with ATP and adenosine alone and in the presence of suramin and 8-sulphophenyltheophylline, showed increased $PGF_{2\alpha}$ output from the

endometrium and the myometrium and increased 6-keto-$PGF_{1\alpha}$ output from the myometrium after 2 h of culture. Increased $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs in the presence of ATP and adenosine after 2 h of culture were not seen in previous culture experiments and the reason for this is not clear. Some inhibition of the ATP-induced stimulation of $PGF_{2\alpha}$ output was observed in the presence of suramin from endometrium after 24 h of culture. However, suramin was also seen to decrease $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from endometrium and myometrium when there was no ATP-induced stimulation and also when in the presence of adenosine. This suggests that, in long-term culture experiments, suramin may be inhibiting $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs by itself. Since suramin alone was not included in the experimental set-up, it is not clear whether suramin really does have any significant effect on PG output from guinea-pig uterus after 24 h of culture. ATP plus 8-sulphophenyltheophylline or adenosine plus 8-sulphophenyltheophylline had very little inhibitory effect on PG production by endometrium and myometrium in 24 h culture experiments when compared to the effect of adenosine alone. This suggests that this effect of adenosine may not be mediated via A receptors in the guinea-pig uterus during 24 h of culture.

ATP and adenosine were observed to decrease PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from guinea-pig endometrium and myometrium, particularly between 2 and 24 h of culture. The same effect on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig endometrium and myometrium were observed in previous culture experiments in the presence of ATP, especially from the myometrium, and were attributed to an increase in the channelling of PGH_2 towards the $PGF_{2\alpha}$ synthesising pathway.

Suramin and 8-sulphophenyltheophylline had little effect on ATP and adenosine-induced increases in PG outputs after 24 h of culture. This suggests that ATP and adenosine-induced increases in PG production were non-receptor-mediated events. However, the effect of suramin on PG output, particularly $PGF_{2\alpha}$ output remains unclear and P2 receptor-mediated stimulation of $PGF_{2\alpha}$ cannot be ruled out.

These present findings support the view that adenosine, ATP and it analogues stimulate PG output (particularly $PGF_{2\alpha}$) from the superfused guinea-pig uterus and from guinea-pig endometrium and myometrium cultured for 24 h. This was more pronounced in the endometrium which is the main site of prostanoid synthesis in the guinea-pig uterus (Poyser, 1983). Scheimann *et al.* (1991) and Suzuki (1991) both observed increased $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the uterus from guinea-pig and rabbit respectively, following treatments with A and P2 agonists. The increase in $PGF_{2\alpha}$ output could be responsible for stimulating uterine contraction in the guinea-pig uterus as suggested by Smith *et al.* (1988) and Piper & Hollingsworth (1996). The effects of the P2 antagonist, suramin, on ATP stimulated $PGF_{2\alpha}$ production from superfused guinea-pig uterus suggests that ATP exerts its effect via P2 receptors. A and P2 receptors have previously been observed in guinea-pig uterus (Smith *et. al.*, 1988). However, as well as acting on P2 receptors, ATP may indirectly be stimulating adenosine receptors after being broken down to adenosine by ecto-nucleotidases especially after 24 h of culture. However, the A receptor antagonist, 8-sulphophenyltheophylline, had no effect on ATP-induced $PGF_{2\alpha}$ output from guinea-pig uterus suggesting that ATP was exerting its action predominantly by activating P2 receptors.

Previous workers have disagreed over the involvement of PGs in spasm of the guinea-pig uterus mediated by A receptors (Scheimann *et* al., 1991; Bradley *et al.*, 1993). Scheimann *et al.* (1991) proposed that cyclooxygenase products are involved in adenosine-induced contractions of the non-pregnant guinea-pig uterus, while Bradley *et al.* (1993) dispute this fact. The present study indicates that PGs may be involved in adenosine-induced spasm of the guinea-pig uterus, as adenosine was observed to stimulate $PGF_{2\alpha}$ output from guinea-pig uterus superfused *in vitro* and this stimulation by adenosine was inhibited by the A receptor antagonist, 8-sulphophenyltheophylline. Therefore, it is clear that ATP- and adenosine-induced increases in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus are receptor mediated responses as the actions of ATP and adenosine were significantly reduced by the P2 and A receptor antagonists, suramin and 8-sulphophenyltheophylline respectively. P2Y purinoceptors have previously been observed in the guinea-pig uterus and more specifically in the endometrium (Piper & Hollingsworth). P2Y receptors are G protein-linked receptors (Webb *et al.*, 1993). They have been observed to couple via G proteins to phospholipase A_2 (PLA_2) (see Axelrod *et al.*, 1988) and phospholipase C (PLC) in a number of tissues (Piper & Hollingsworth, 1996). PLA_2 is well documented as the enzyme responsible for arachidonic acid release from membrane phospholipids, and it is possibly responsible for increased PG synthesis from the superfused guinea-pig uterus stimulated by adenosine, ATP and ATP analogues. However, PLC can also mobilise arachidonic acid from inositol phospholipids (IP). Diacylglycerol (DAG) is one of the breakdown products of PI hydrolysis. Arachidonic acid can be released from DAG by the sequential action of diacylglycerol lipase and monoacylglycerol lipase. Previous workers have suggested

that adenosine and ATP-induced stimulation of PG synthesis could be linked to IP hydrolysis in the guinea-pig and rabbit uterus (Scheimann *et al.*, 1991; Suzuki, 1991). ATP has been observed to stimulate DAG formation, indicative of PLC activation (Suzuki, 1991) and stimulation of adenosine receptors leads to rapid accumulation of inositol phosphates (Scheimann *et al.*, 1991).

Diacylglycerol (DAG) can also activate protein kinase C (PKC), another second messenger system that has been implicated in stimulation of PGHS expression (DeWitt, 1991), which can stimulate a kinase cascade leading to mitogen-activated protein kinase (MAPK) activation (Qiu & Leslie, 1994). MAPK can activate transcription factors (Karin & Hunter, 1995), and in particular NF-κB which leads to induction of expression of many immediate early genes (Baeuerle, 1991). NF-κB has been observed to increase PGHS-2 synthesis in lipopolysaccharide (LPS)-stimulated macrophages (Hwang *et al.*, 1997). This may be the mechanism for increased PGHS synthesis by the guinea-pig uterus observed after 24 h of culture. Previous studies have shown the presence of PGHS-2 in the guinea-pig endometrium by western blotting (Naderali & Poyser, 1994) and have demonstrated that PGHS-2 is the main functional form of PGHS in the guinea-pig endometrium and myometrium (Naderali & Poyser, 1996a).

MAPK also activates $cPLA_2$ (Lin *et al.*, 1993) which could also result in increased PG synthesis. Inositol 1,4,5-triphosphate (IP_3) is a specific breakdown product of PLC-mediated PIP_2 hydrolysis and is a unique second messenger that releases calcium from the endoplasmic reticulum of several cell types, including the sarcoplasmic reticulum of smooth muscle cells (Schrey *et al.*, 1988). Scheimann *et al.* (1991) suggest that IP_3 is capable of releasing calcium from the guinea-pig

uterine intracellular stores. Increased calcium concentration can activate PLA_2 enzymes and stimulate arachidonic acid mobilisation and increased PG synthesis. Therefore, there are a number of possible mechanisms for arachidonic acid release and stimulation of PG synthesis in the guinea-pig uterus by ATP, ATP analogues and adenosine. Further studies are required to elucidate which pathways are actually involved.

3B. A STUDY OF PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG PLACENTA

3B.1 BASAL PROSTAGLANDIN PRODUCTION AND THE EFFECTS OF PROSTAGLANDIN H SYNTHASE (PGHS) INHIBITORS

Introduction:

The placenta is a major site of prostaglandin (PG) biosynthesis during pregnancy (Keirse, 1979). PG production by the placenta has been reported to increase during pregnancy and particularly towards parturition in many animal species (Challis & Olson, 1988; Mitchell, 1988). PG production by the guinea-pig placenta and sub-placenta has been investigated during early to mid pregnancy. The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured on day 22 and day 29 of gestation following a 24 h culture period. $PGF_{2\alpha}$ can be metabolised to 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM). The output of this metabolite was also measured to determine whether any increase in $PGF_{2\alpha}$ output from guinea-pig placenta was accompanied by an increase in $PGF_{2\alpha}$ metabolism.

Prostaglandins of the 2-series are synthesised from arachidonic acid and the first step in this synthesis is the conversion of arachidonic acid to PGH_2 by prostaglandin H synthase (PGHS). Recently two PGHS enzymes have been identified, cloned and sequenced (DeWitt & Smith, 1988; Merlie *et al.*, 1988; Kujubu *et al.*, 1991;

O'Banion *et al.*, 1992): they are called PGHS-1 and PGHS-2. This raises the question as to which form is responsible for PG production by the guinea-pig placenta and sub-placenta? Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase activity of PGHS. The effects of indomethacin, a non-selective inhibitor of PGHS, and the PGHS-2 selective inhibitor NS-398 (N(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide), have been investigated. Both compounds have similar potencies at inhibiting PGHS-2 but, in contrast to indomethacin, NS-398 has no inhibitory effect on PGHS-1 (Futaki *et al.*, 1993, 1994). It has also been reported that indomethacin is more potent at inhibiting PGHS-1 than PGHS-2 (Mitchell *et al.*, 1993). The effects of indomethacin and NS-398 on PG output from day 22 and day 29 guinea-pig placenta and sub-placenta cultured for 24 h were observed. In addition, the effects of these two inhibitors on PG production by homogenates of guinea-pig placenta on days 22 and 29 of pregnancy were studied.

Methods:

In tissue culture experiments, the placentae of day 22 and 29 pregnant guinea-pigs were removed and separated as described in Section 2.1.4. The placenta and sub-placenta were cut into small pieces approximately 1 mm^3, and placed on lens tissue on the top of raised, gauze platforms in culture dishes. Each dish contained 4 ml tissue culture medium (TCM). In experiment 3B.1.1, day 22 and 29 placenta and sub-placenta were cultured in TCM only, in order to obtain basal PG outputs. Experiments 3B.1.3-5 were carried out in the presence of indomethacin (5, 20 and 40 μM) or NS-398 (5, 20 and 40 μM) which was added to the TCM in each Petri dish. The Petri dishes were placed in modified Kilner jars, which were gassed and

incubated as previously described (Section 2.1.2). Culture medium was removed after 2, 8 and 24 h. The samples of culture medium were stored at -20°C and assayed for their $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ content by radioimmunoassay (RIA). Enzyme-linked immunosorbent assay (ELISA) was used to measure PGFM content in the culture medium obtained from day 22 and 29 control placenta. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Homogenisation experiments were carried out using the placenta from day 29 pregnant guinea-pigs. Tissue was removed and prepared as described in Section 2.1.4. The tissue was blotted dry, weighed and then homogenised as described in Section 2.1.3 with Krebs solution containing either indomethacin (40 μM) or NS-398 (40 μM). One sample in each experiment was untreated and acted as a control. Each sample was allowed to incubate for 60 min in a water bath at 37°C (see Section 2.1.3), and prostaglandins were then extracted as described in Section 2.1.1. Samples were evaporated to dryness on a rotary evaporator, re-dissolved in 5 ml ethyl acetate and stored at -20°C until assayed for their PG content. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Results:

Experiment 3B.1.1 Prostaglandin Output from Day 22 and 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

The outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from guinea-pig placenta decreased significantly ($P < 0.05$, n=5) with time during the 24 h culture period on both days 22 and 29 of pregnancy (Figs. 3B.1.1.1a, b and c). $PGF_{2\alpha}$ and 6-keto-$F_{1\alpha}$ outputs from day 29 guinea-pig placenta were significantly ($P < 0.05$, n=5) higher than $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the day 22 guinea-pig placenta, respectively, throughout the 24 h culture period (Figs. 3B.1.1.1a and c). PGE_2 output from guinea-pig placenta did not change significantly between days 22 and 29 of pregnancy throughout culture (Fig. 3B.1.1.1b).

Sub-Placenta

The outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from guinea-pig sub-placenta decreased significantly ($P < 0.05$, n=5) with time during the 24 h culture period on both days 22 and 29 of pregnancy (Figs. 3B.1.1.2a, b and c). $PGF_{2\alpha}$ output from day 22 guinea-pig placenta was not significantly different from $PGF_{2\alpha}$ output from day 22 sub-placenta (Figs. 3B.1.1.1, 3B.1.1.2a and c). PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 22 placenta were significantly ($P < 0.05$, n=5) higher than PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 22 sub-placenta, except 6-keto-$PGF_{1\alpha}$ output after 2 h

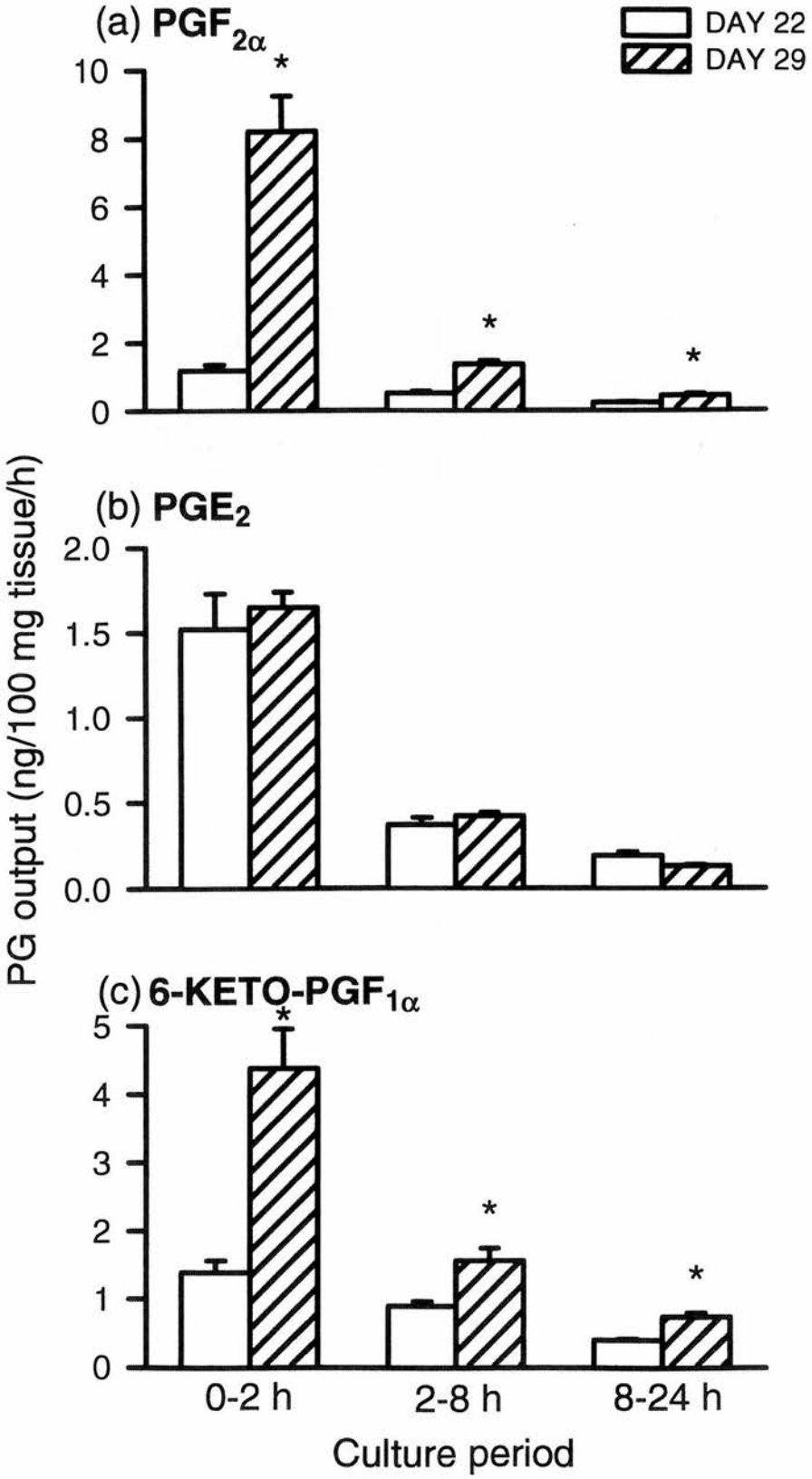


Figure 3B.1.1.1 Mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 22 and 29 guinea-pig placenta cultured for 24 h.
* Significantly higher, $P < 0.05$, than corresponding day 22 value.

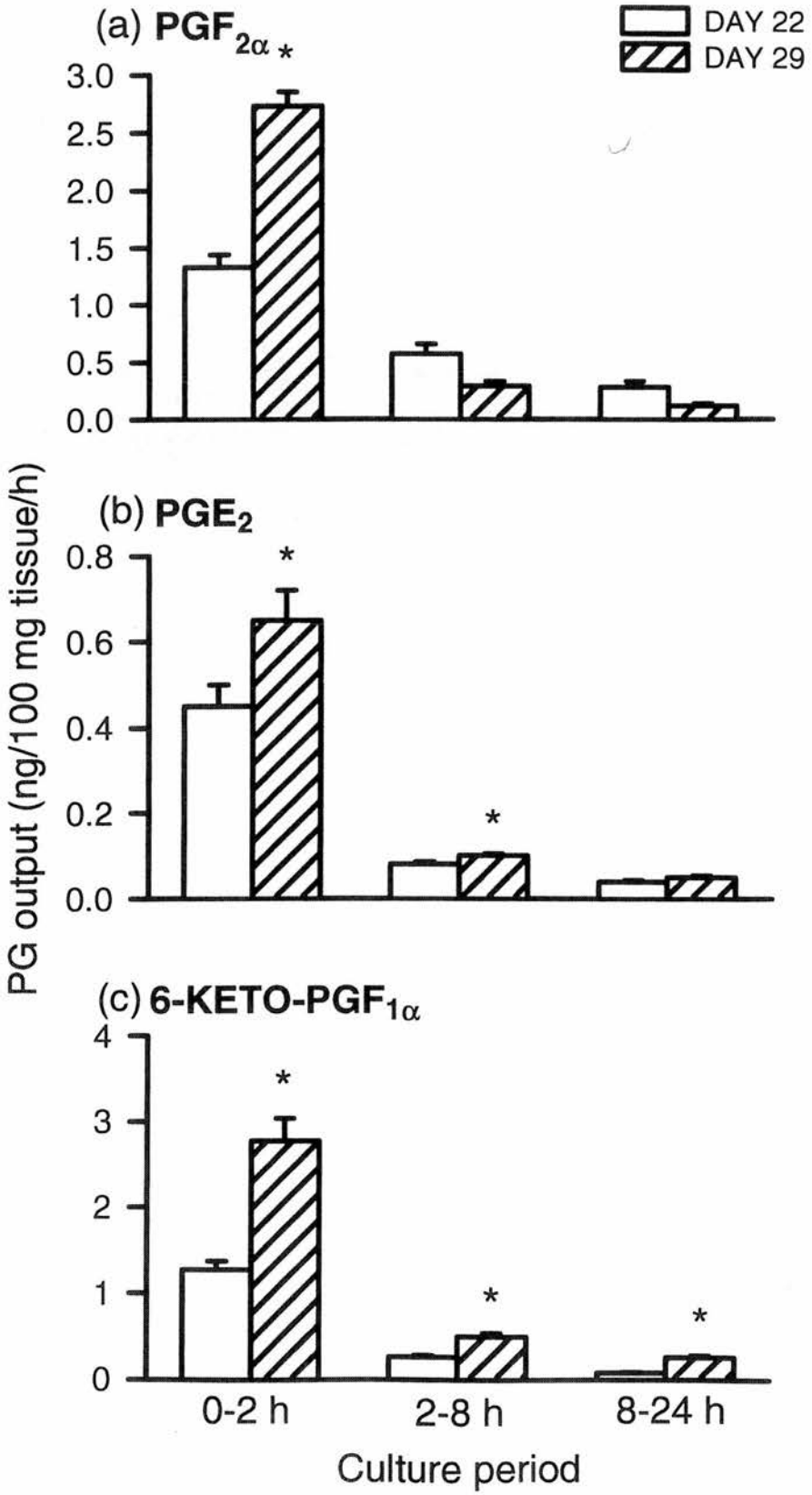


Figure 3B.1.1.2 Mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 22 and day 29 guinea-pig sub-placenta cultured for 24 h.

* Significantly higher, $P < 0.05$, than corresponding day 22 value.

of culture (Fig. 3B.1.1.2b). $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 29 guinea-pig placenta were significantly ($P < 0.05$, n=5) higher than $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 29 sub-placenta, 6-keto-$PGF_{1\alpha}$ output after 2 h of culture (Figs. 3B.1.1.1, 3B.1.1.2a, b and c). $PGF_{2\alpha}$, output from day 29 guinea-pig sub-placenta was significantly ($P < 0.05$, n=5) increased after 2 h of culture but not after 8 and 24 h, when compared to $PGF_{2\alpha}$ output from day 22 sub-placenta (Fig. 3B.1.1.2a). PGE_2 output was significantly ($P < 0.05$, n=5) higher on day 29 of pregnancy after 2 h and 8 h of culture but not after 24 h (Fig. 3B.1.1.2b). 6-Keto-$F_{1\alpha}$ output from day 29 sub-placenta was significantly ($P < 0.05$, n=5) higher throughout the 24 h culture period compared to 6-keto-$PGF_{1\alpha}$ output from day 22 guinea-pig sub-placenta (Fig. 3B.1.1.2c).

Experiment 3B.1.2 PGFM Output from Day 22 and 29 Guinea-Pig Placenta.

The output of PGFM from guinea-pig placenta decreased with time during the culture period on both day 22 and day 29 of pregnancy (Fig. 3B.1.2.1). PGFM output from the guinea-pig placenta was significantly ($P < 0.05$, n=5) higher on day 29 of pregnancy compared to day 22 during the first 2 h of culture, but not after 8 h and 24 h of culture (Fig. 3B.1.2.1). $PGF_{2\alpha}$ output from day 29 placenta was 8-fold higher compared to $PGF_{2\alpha}$ output from day 22 placenta during the first 2 h of culture. PGFM output from day 29 placenta was 2.5-fold higher compared to PGFM output from day 22 placenta after 2 h of culture (Fig. 3B.1.1.1a and 3B.1.2.1). After 8 h of

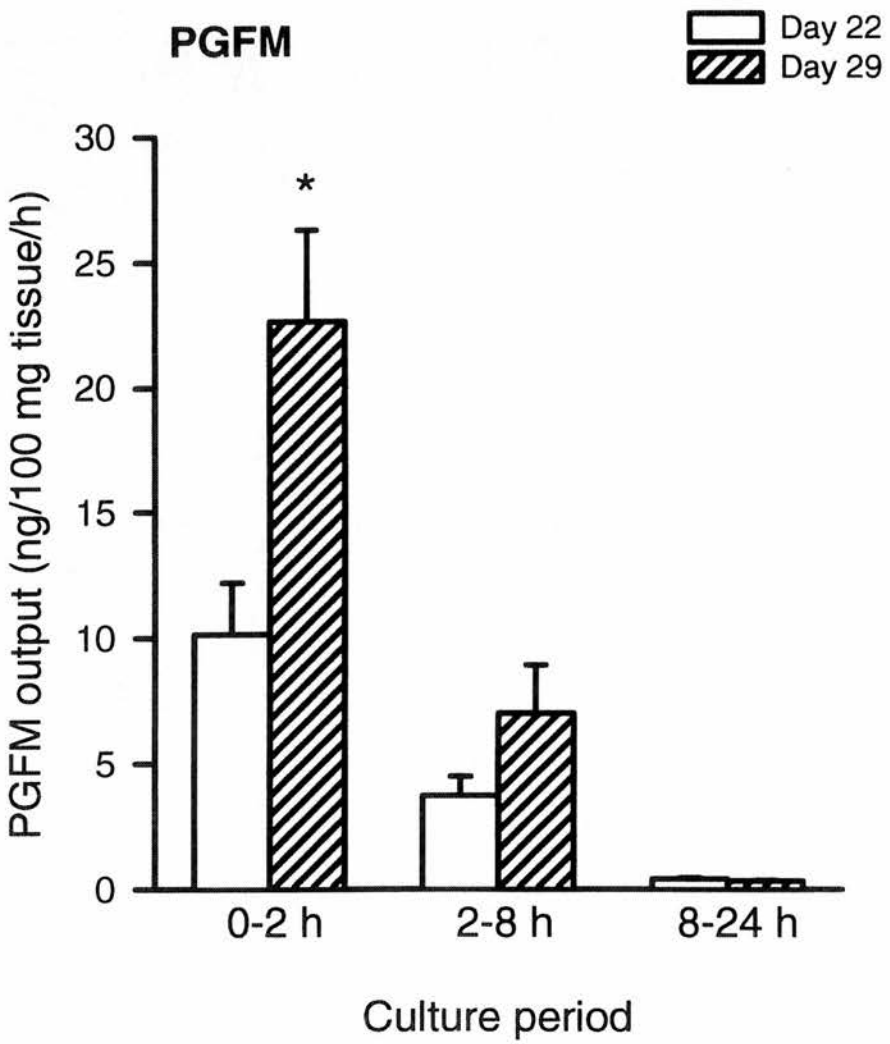


Figure 3B.1.2.1 Mean (± SEM, n=5) output of PGFM from day 22 and 29 guinea-pig placenta cultured for 24 h.
*Significantly higher, $P < 0.05$, than corresponding day 22 value.

culture $PGF_{2\alpha}$ and PGFM outputs from day 29 placenta had increased 2-fold compared to outputs from day 22 placenta (Figs. 3B.1.1.1a and 3B.1.2.1). After 24 h of culture, there was very little difference between the output of PGFM from day 22 and 29 placenta, while $PGF_{2\alpha}$ output from day 29 guinea-pig placenta was significantly ($P < 0.05$, n=5) higher than the day 22 $PGF_{2\alpha}$ output (Figs. 3B.1.1.1a and 3B.1.2.1).

Experiment 3B.1.3 The Effects of Indomethacin and NS-398 on PG Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

Indomethacin (20 μM, but not 5 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output from day 29 guinea-pig placenta after 2 h of culture (Fig. 3B.1.3.1a). After 8 and 24 h of culture, indomethacin (5 and 20 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output (Fig. 3B.1.3.1a). NS-398 (5 and 20 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output throughout the 24 h culture period (Fig. 3B.1.3.1a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (20 μM, but not 5 μM) when compared to the corresponding indomethacin treatment after 24 h of culture but not after 2 and 8 h (Fig. 3B.1.3.1a). PGE_2 output from day 29 guinea-pig placenta was significantly ($P < 0.05$, n=5) reduced by indomethacin (20 μM, but not 5 μM) and NS-398 (20 μM, but not 5 μM) after 2 h, and by indomethacin (5 and 20 μM) after 8 h but not after 24 h (Fig. 3B.1.3.1b). NS-398 (5

and 20 μM) had no effect on PGE_2 output from day 29 guinea-pig placenta after 8 h and 24 h of culture (Fig. 3B.1.3.1b). There was no significant difference between PGE_2 output from the placenta in the presence of indomethacin (5 and 20 μM) compared to PGE_2 output in the presence of NS-398 (5 and 20 μM) (Fig. 3B.1.3.1b). Indomethacin (5 and 20 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$F_{1\alpha}$ output from day 29 placenta throughout the 24 h culture period (Fig. 3B.1.3.1c). NS-398 (5 and 20 μM) significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output in the first 2 h of culture but not after 8 h (Fig. 3B.1.3.1c). After 24 h of culture, NS-398 (5 μM, but not 20 μM) significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output (Fig. 3B.1.3.1c). 6-Keto-$F_{1\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (20 μM, but not 5 μM) when compared to treatment with indomethacin (20 μM) after 8 and 24 h of culture but not in the first 2 h (Fig. 3B.1.3.1c).

Sub-Placenta

$PGF_{2\alpha}$ output from day 29 guinea-pig sub-placenta was significantly ($P < 0.05$, n=5) reduced by indomethacin (5 and 20 μM) after 2 and 24 h of culture, but not after 8 h of culture (Fig. 3B.1.3.2a). NS-398 (5 and 20 μM) had no effect on $PGF_{2\alpha}$ output, except NS-398 (20 μM) which significantly ($P < 0.05$, n=5) decreased $PGF_{2\alpha}$ output after 24 h of culture (Fig. 3B.1.3.2a). $PGF_{2\alpha}$ output in the presence of NS-398 (20 μM, but not 5 μM) was significantly ($P < 0.05$, n=5) higher than $PGF_{2\alpha}$ output in the presence of indomethacin (20 μM, but not 5 μM) (Fig. 3B.1.3.2a). Indomethacin (5 μM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output from day 29 sub-placenta

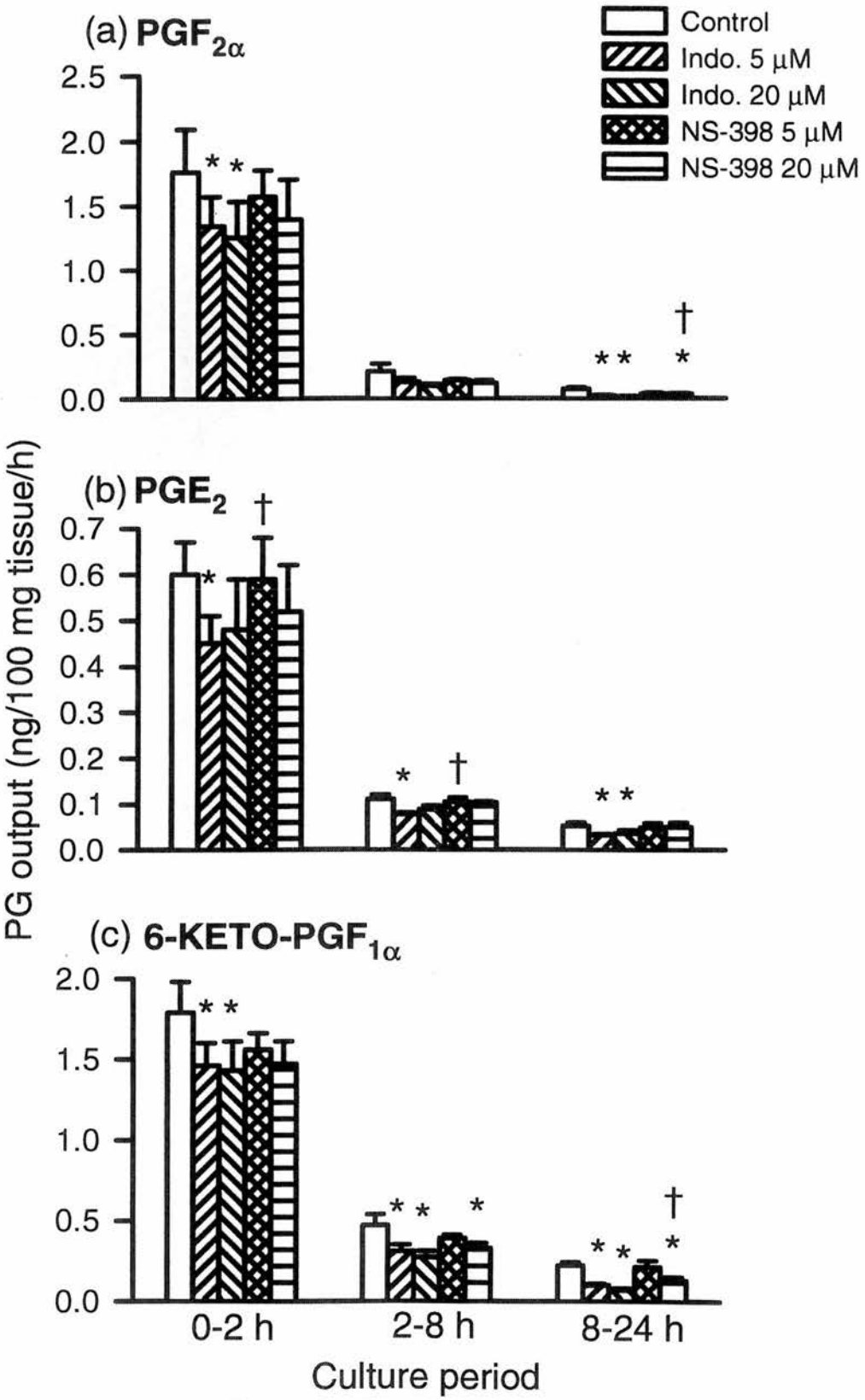


Figure 3B.1.3.2 Effects of indomethacin (5 and 20 μM) and NS-398 (5 and 20 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.

*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than corresponding value obtained from indomethacin treatment.

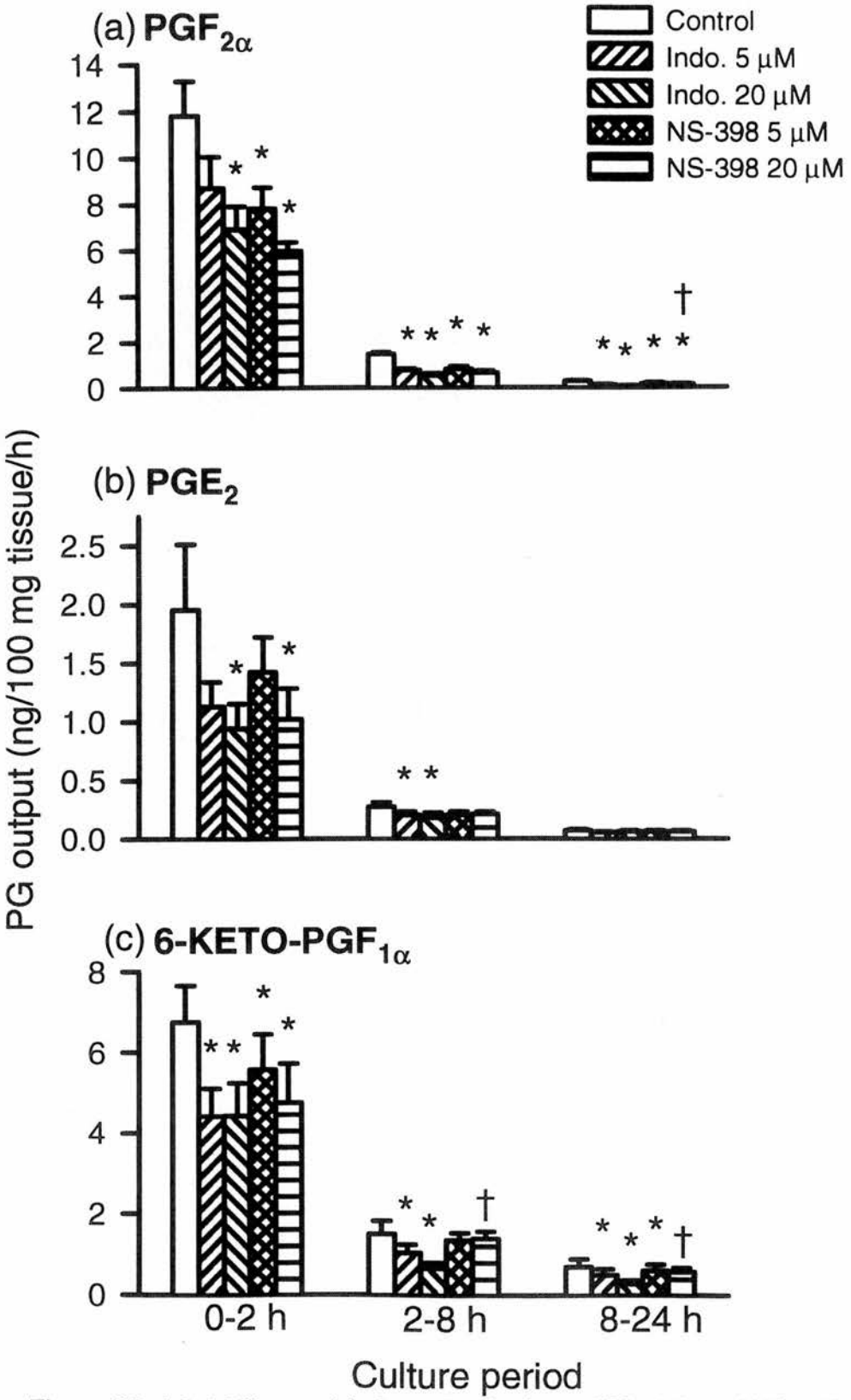


Figure 3B.1.3.1 Effects of indomethacin (5 and 20 μM) and NS-398 (5 and 20 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h

*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than corresponding value obtained from indomethacin treatment.

throughout the 24 culture period; indomethacin (20 μM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output after 24 h, but not after 2 and 8 h of culture (Fig. 3B.1.3.2b). NS-398 (5 and 20 μM) had no significant effect on PGE_2 output (Fig. 3B.1.3.2b) from day 29 guinea-pig sub-placenta. PGE_2 output was significantly greater ($P < 0.05$, n=5) in the presence of NS-398 (5 μM, but not 20 μM) after 2 and 8 h but not after 24 h of culture, when compared to the corresponding value obtained for indomethacin treatment (Fig. 3B.1.3.2b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) reduced throughout the 24 h culture period by indomethacin (5 and 20 μM) and by NS-398 (20 μM, but not 5 μM) after 8 h and 24 h but not in the first 2 h of culture (Fig. 3B.1.3.2c). 6-Keto-$PGF_{1\alpha}$ output in the presence of NS-398 (20 μM, but not 5 μM) was significantly ($P < 0.05$, n=5) higher than 6-keto-$PGF_{1\alpha}$ output in the presence of indomethacin (20 μM) after 24 h but not after 2 and 8 h of culture (Fig. 3B.1.3.2c).

Experiment 3B.1.4 The Effects of Indomethacin (40 μM) and NS-398 (40 μM) on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

Indomethacin (40 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ throughout the 24 h culture period (Fig. 3B. 1.4.1a). NS-398 (40 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output after 24 h, but not after 2 and 8 h of culture (Fig. 3B. 1.4.1a).

After 8 and 24 h of culture, but not after 2 h, $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (40 μM) when compared to $PGF_{2\alpha}$ output in the presence of indomethacin (40 μM) (Fig. 3B. 1.4.1a). PGE_2 output was reduced significantly ($P < 0.05$, n=5) by indomethacin (40 μM) throughout the 24 h culture period (Fig. 3B.1.4.1b). NS-398 (40 μM) significantly ($P < 0.05$, n=5) decreased PGE_2 output after 2 and 8 h of culture, but had no effect after 24 h (Fig. 3B.1.4.1b). Throughout the 24 h culture period, PGE_2 output was significantly ($P < 0.05$, n=5) greater in the presence of NS-398 (40 μM) when compared to the PGE_2 output in the presence of indomethacin (40 μM) (Fig. 3B.1.4.1b). 6-Keto-$PGF_{1\alpha}$ output was reduced significantly ($P < 0.05$, n=5) by indomethacin (40 μM) throughout the 24 h culture period, and by NS-398 (40 μM) after 2 and 8 h, but not after 24 h of culture (Fig. 3B.1.4.1c). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) higher after 8 and 24 h, but not in the first 2 h, in the presence of NS-398 (40 μM) when compared to the corresponding value obtained for indomethacin (40 μM) treatment (Fig. 3B.1.4.1c).

Sub-Placenta

$PGF_{2\alpha}$ output from day 29 sub-placenta was significantly ($P < 0.05$, n=5) decreased by indomethacin (40 μM) and NS-398 (40 μM) throughout the 24 h culture period (Fig. 3B.1.4.2a). After 8 h, but not after 2 and 24 h of culture, $PGF_{2\alpha}$ output in the presence of NS-398 (40 μM) was significantly ($P < 0.05$, n=5) higher than $PGF_{2\alpha}$ output in the presence of indomethacin (40 μM) (Fig. 3B.1.4.2a). Indomethacin (40 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output throughout the 24 h culture

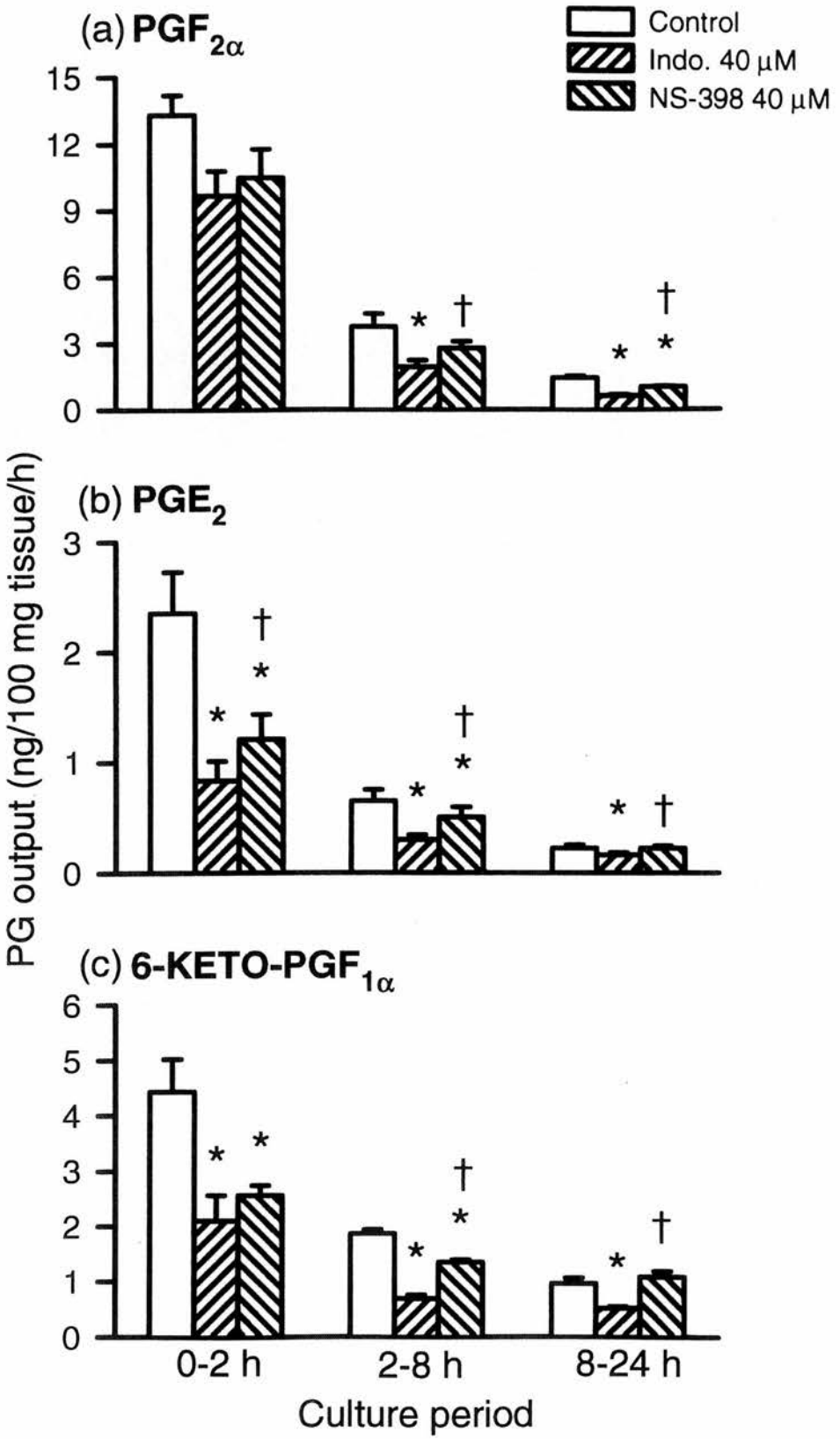


Figure 3B.1.4.1 Effects of indomethacin (40 μM) and NS-398 (40 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.
*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than corresponding value obtained from indomethacin treatment.

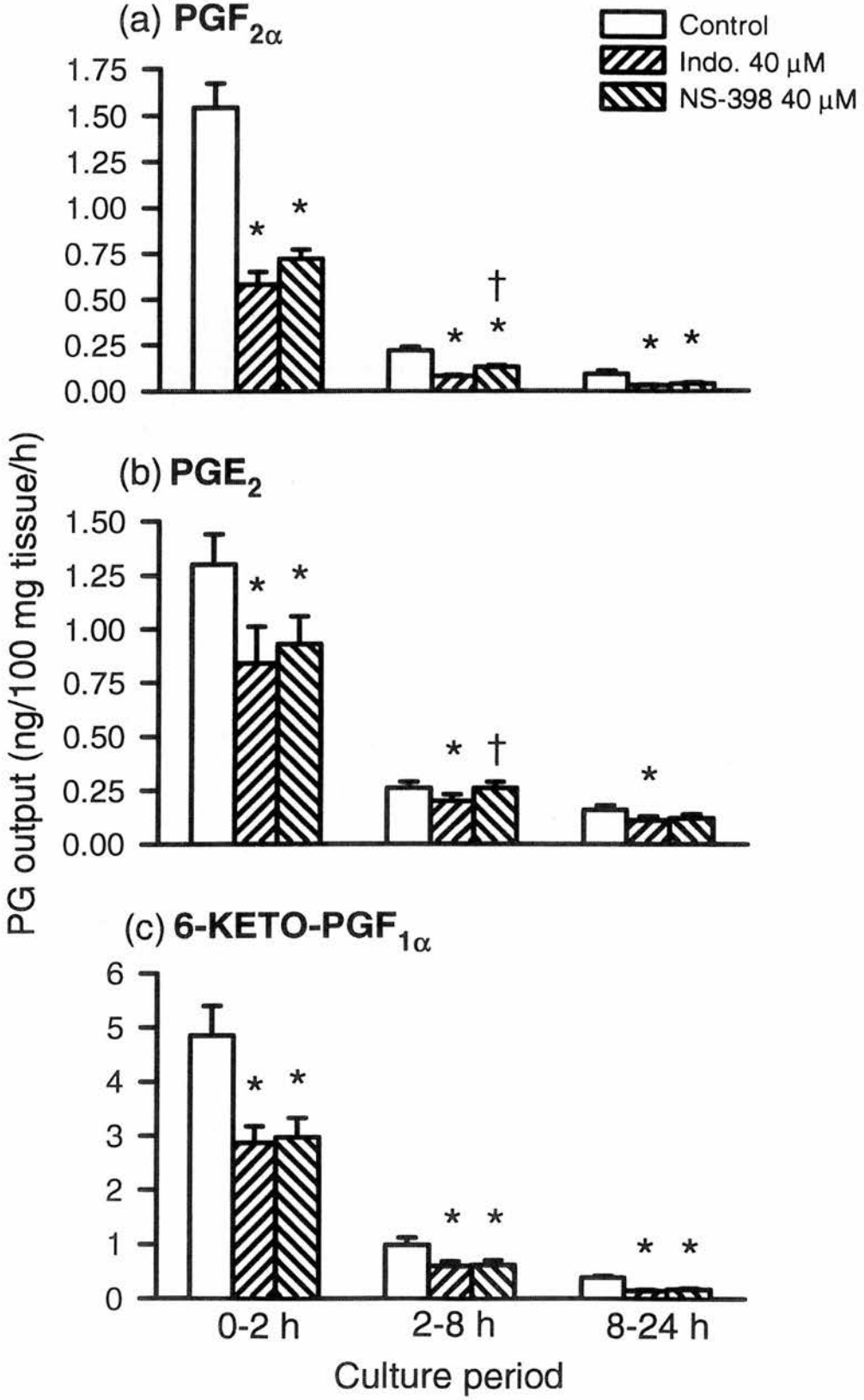


Figure 3B.1.4.2 Effects of indomethacin (40 μM) and NS-398 (40 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.
*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than corresponding value obtained from indomethacin treatment.

period (Fig. 3B.1.4.2b). NS-398 (40 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output after 2 h of culture, but not after 8 and 24 h (Fig. 3B.1.4.2b). PGE_2 output in the presence of NS-398 (40 μM) was significantly ($P < 0.05$, n=5) greater than PGE_2 output in the presence of indomethacin (40 μM) after 8 h, but not after 2 h and 24 h of culture (Fig. 3B.1.4.2b). 6-Keto-$F_{1\alpha}$ output was inhibited significantly ($P < 0.05$, n=5) throughout the 24 h culture period by indomethacin (40 μM) and by NS-398 (40 μM) (Fig. 3B.1.4.2c). There were no significant differences when 6-keto-$PGF_{1\alpha}$ output from the sub-placenta in the presence NS-398 (40 μM) was compared to the corresponding output in the presence of indomethacin (40 μM) (Fig. 3B.1.4.2c).

Experiment 3B.1.5 The Effect of Indomethacin and NS-398 on Prostaglandin Output from Day 22 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

Indomethacin (5, 20 and 40 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ throughout the 24 h culture period (Fig. 3B.1.5.1a). NS-398 (5, 20 and 40 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.1.5.1a). After 8 h, NS-398 (40 μM, but not 5 and 20 μM) significantly ($P < 0.05$, n=5) decreased $PGF_{2\alpha}$ output and, after 24 h, $PGF_{2\alpha}$ output was reduced significantly ($P < 0.05$, n=5) by NS-398 (5 and 40 μM, but not 20 μM) (Fig. 3B.1.5.1a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (40 μM, but not 5 and 20 μM) after 8 h, but not after 2 and 24 h of culture, when compared to

the corresponding indomethacin (40 μM) treatment (Fig. 3B.1.5.1a). PGE_2 output was reduced significantly ($P < 0.05$, n=5) by indomethacin (5, 20 and 40 μM) and by NS-398 (5, 20 and 40 μM) throughout the 24 h culture period, except for 20 μM NS-398 after 8 h (Fig. 3B.1.5.1b). PGE_2 output from day 22 guinea-pig placenta was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (5 and 40 μM, but not 20 μM) when compared to PGE_2 output in the presence of the corresponding indomethacin concentration (5 and 40 μM, but not 20 μM) treatments after 2 and 8 h (Fig. 3B.1.5.1b). After 24 h of culture, PGE_2 output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (40 μM, but not 5 and 20 μM) compared to output in the presence of indomethacin (40 μM, but not 5 and 20 μM) (Fig. 3B.1.5.1b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) reduced by indomethacin (5 and 40 μM, but not 20 μM) in the first 2 h of culture and by indomethacin (5, 20 and 40 μM) after 8 h (Fig. 3B.1.5.1c). After 24 h, 6-keto-$PGF_{1\alpha}$ output was reduced significantly ($P < 0.05$, n=5) by indomethacin (40 μM, but not 5 and 20 μM) (Fig. 3B.1.5.1c). NS-398 (5, 20 and 40 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output, except for 40 μM NS-398 which significantly ($P < 0.05$, n=5) decreased output after 2 and 8 h of culture, and 5 μM NS-398 which reduced 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 24 h (Fig. 3B.1.5.1c). After 8 h, but not after 2 and 24 h of culture, 6-keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (20 and 40 μM, but not 5 μM) when compared to the corresponding 6-keto-$PGF_{1\alpha}$ output in the presence of indomethacin (20 and 40 μM, but not 5 μM) (Fig. 3B.1.5.1c).

Sub-Placenta

$PGF_{2\alpha}$ output from day 22 sub-placenta was inhibited significantly ($P < 0.05$, n=5) by indomethacin (5, 20 and 40 μM) and by NS-398 (5, 20 and 40 μM) throughout the 24 h culture period (Fig. 3B.1.5.2a). After 2 and 24 h of culture, $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (20 μM, but not 5 and 40 μM) when compared to $PGF_{2\alpha}$ output in the presence of indomethacin (20 μM, but not 5 and 40 μM) (Fig. 3B.1.5.2a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) higher after 8 h, in the presence of NS-398 (5, 20 and 40 μM) when compared to the corresponding output in the presence of indomethacin (5, 20 and 40 μM) (Fig. 3B.1.5.2a). Indomethacin (5, 20 and 40 μM) and NS-398 (5 and 40 μM, but not 20 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output throughout the culture period (Fig. 3B.1.5.2b). PGE_2 output was significantly ($P < 0.05$, n=5) greater in the presence of NS-398 (20 μM, but not 5 and 40 μM) compared to the corresponding PGE_2 output in the presence of indomethacin (20 μM, but not 5 and 40 μM) throughout the culture period (Fig. 3B.1.5.2b). 6-Keto-$F_{1\alpha}$ output was inhibited significantly ($P < 0.05$, n=5) by indomethacin (5, 20 and 40 μM) throughout the 24 h culture period (Fig. 3B.1.5.2c). NS-398 (40 μM, but not 5 and 20 μM) significantly ($P < 0.05$, n=5) decreased 6-keto-$PGF_{1\alpha}$ output after 2 h of culture, and NS-398 (5 and 40 μM, but not 20 μM) decreased 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 8 and 24 h (Fig. 3B.1.5.2c). 6-Keto-$PGF_{1\alpha}$ output from the sub-placenta in the presence of NS-398 (20 μM, but not 5 and 40 μM) was significantly ($P < 0.05$, n=5) higher after 8 and 24 h, but not after 2 h of culture, when compared to 6-keto-

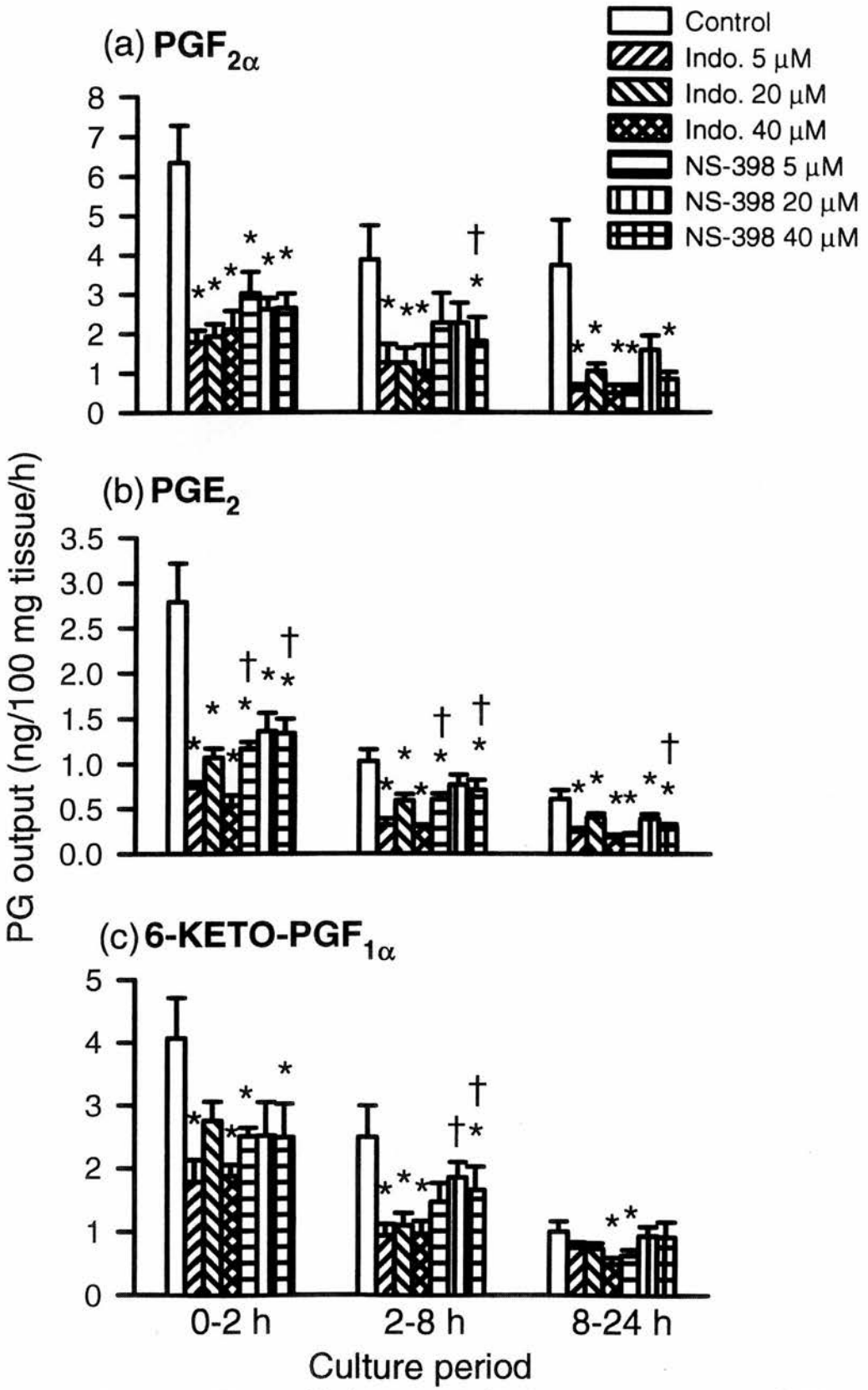


Figure 3B.1.5.1 Effects of indomethacin (5, 20 and 40 μM) and NS-398 (5, 20 and 40 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 22 guinea-pig placenta cultured for 24 h.
*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than corresponding value obtained from indomethacin treatment.

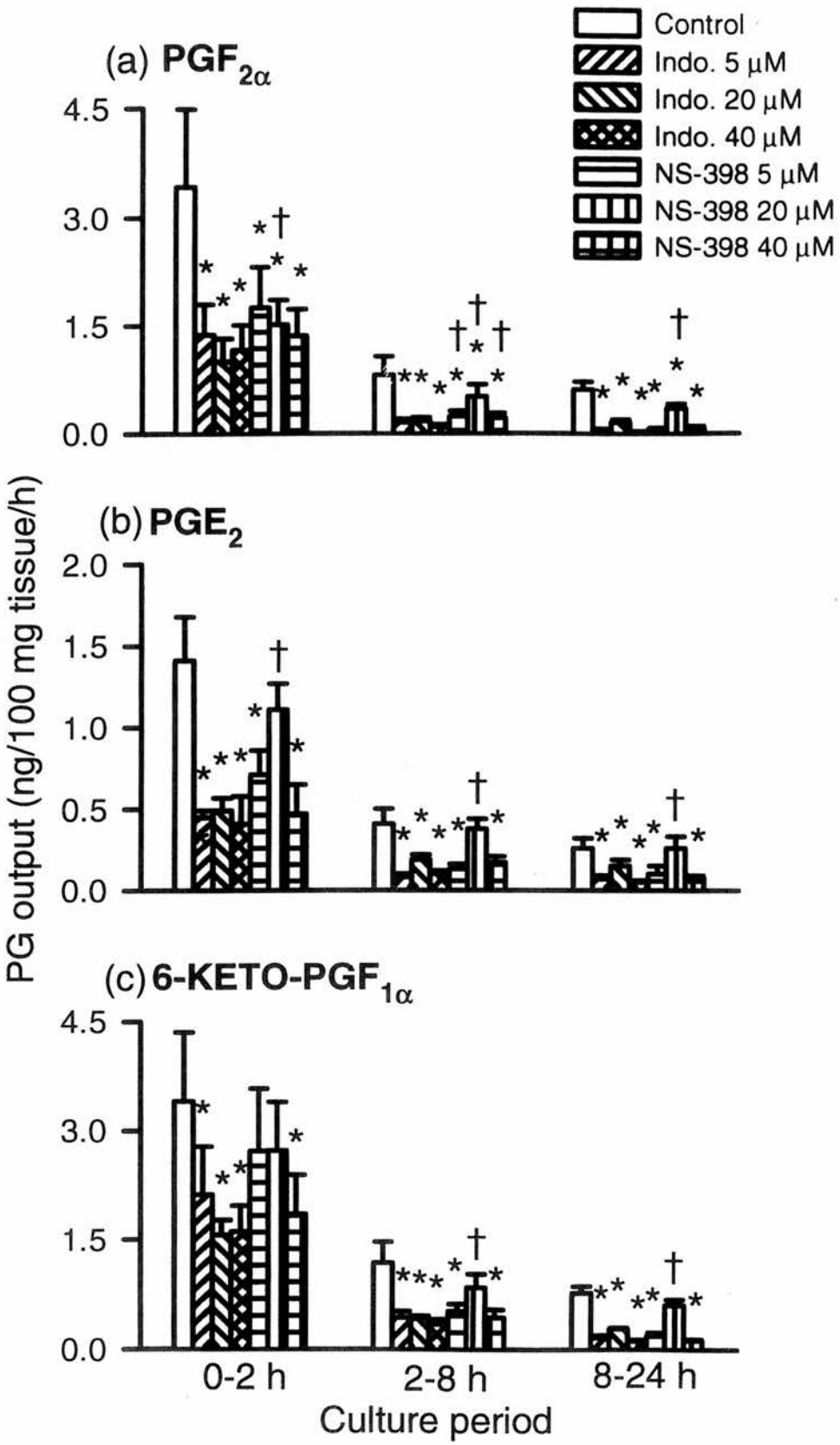


Figure 3B.1.5.2 Effects of indomethacin (5, 20 and 40 μM) and NS-398 (5, 20 and 40 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 22 guinea-pig sub-placenta cultured for 24 h.

*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, P , 0.05, than corresponding value obtained from indomethacin treatment.

$PGF_{1\alpha}$ output in the presence of indomethacin (20 μM, but not 5 and 40 μM) (Fig. 3B.1.5.2c).

Experiment 3B.1.6 The Effects of Indomethacin and NS-398 on Prostaglandin Production by Day 22 and 29 guinea-Pig Placental Homogenates.

Day 22

The amount of $PGF_{2\alpha}$ produced by day 22 guinea-pig placental homogenates was significantly ($P < 0.05$, n=5) greater than the amounts of PGE_2 and 6-keto-$PGF_{1\alpha}$ produced (Fig. 3B.1.6.1a). The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by homogenates of day 22 placenta incubated for 1 h were significantly ($P < 0.05$, n=5) inhibited by indomethacin (40 μM) (Fig. 3A.1.6.1a). The presence of NS-398 (40 μM) had no significant effect on the production of $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ by day 22 placental homogenates although $PGF_{2\alpha}$ production tended to be decreased (Fig. 3A.1.6.1a). The amounts of $PGF_{2\alpha}$ and PGE_2 (but not of 6-keto-$PGF_{1\alpha}$) formed were significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (40 μM) when compared to the corresponding amounts produced in the presence of indomethacin (40 μM) (Fig. 3A.1.6.1a).

Day 29

$PGF_{2\alpha}$ production by day 29 guinea-pig placental homogenates was significantly ($P < 0.05$, n=5) greater than either the production of PGE_2 or 6-keto-$PGF_{1\alpha}$ (Figs.

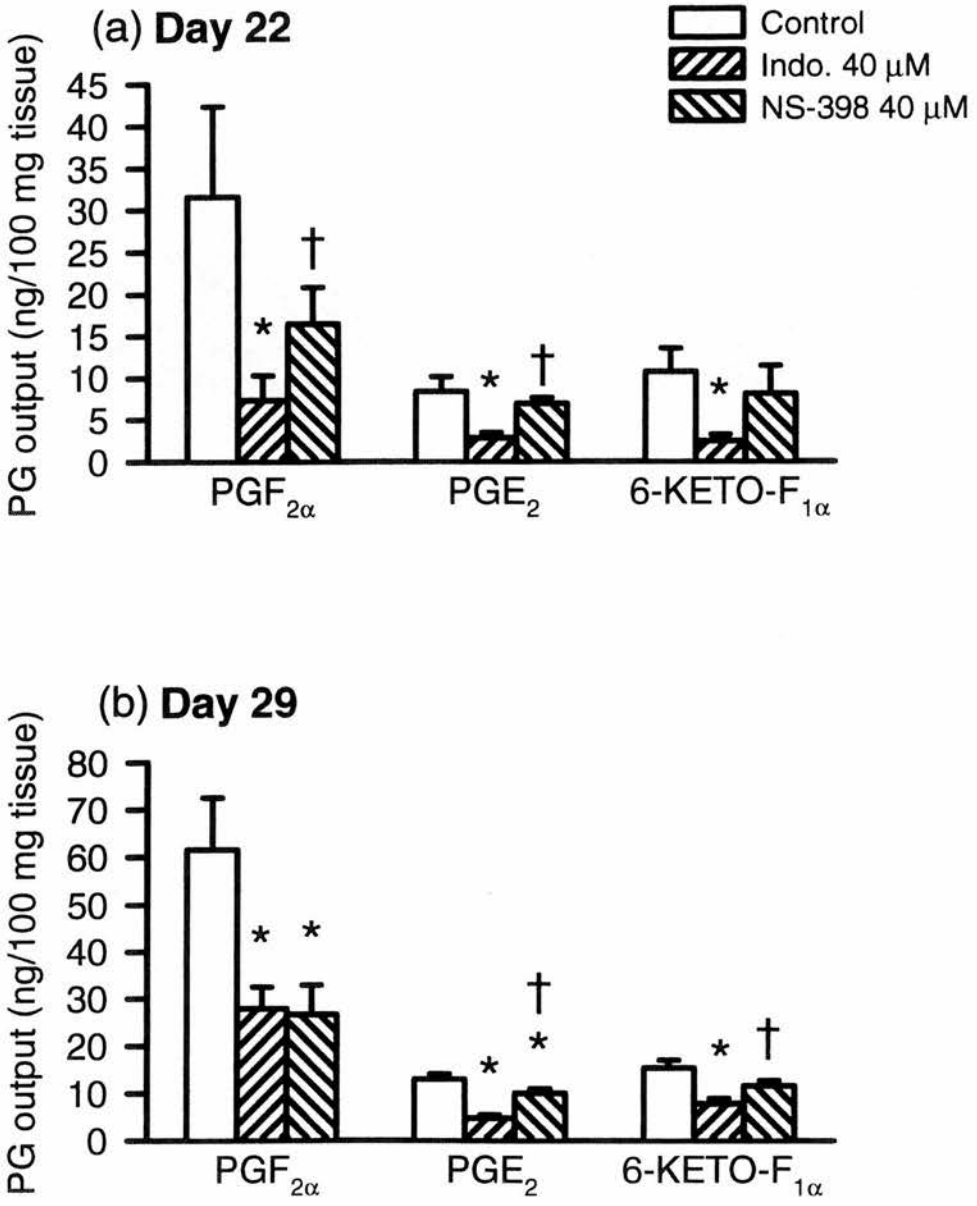


Figure 3B.1.6.1 Effects of indomethacin (40 μM) and NS-398 (40 μM) on mean (±SEM, n=5) amounts of $PGF_{2\alpha}$, PGE_2 and 6-KETO-$F_{1\alpha}$ produced by (a) day 22 and (b) day 29 guinea-pig placenta homogenates following 1 h incubation.

*Significantly lower, $P < 0.05$, than corresponding control value.
†Significantly higher, $P < 0.05$, than correponding value obtained from indomethacin treatment.

3B.1.6.1a and b). The basal amounts of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ produced by day 29 guinea-pig placenta were not significantly different from the amounts of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ produced by day 22 placenta (Figs. 3B.1.6.1a and b). Basal PGE_2 production by day 29 placenta was significantly ($P < 0.05$, n=5) higher than PGE_2 production by day 22 placenta (Figs. 3B.1.6.1a and b). The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by day 29 placental homogenates were significantly ($P < 0.05$, n=5) reduced following 1 h incubation with indomethacin (40 μM) (Fig. 3B.1.6.1b). NS-398 (40 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ and PGE_2 production while having no significant effect on 6-keto-$PGF_{1\alpha}$ production (Fig. 3B.1.6.1b). PGE_2 and 6-keto-$PGF_{1\alpha}$ production (but not $PGF_{2\alpha}$ production) was significantly ($P < 0.05$, n=5) higher in the presence of NS-398 (40 μM) when compared to production in the presence of indomethacin (40 μM) (Fig. 3B.1.6.1b).

Discussion:

This study has shown that $PGF_{2\alpha}$ and 6-keto-$F_{1\alpha}$ outputs from the mid-pregnant day 29 guinea-pig placenta were significantly higher than outputs from day 22 placenta, which is consistent with the work of Csapo *et al.* (1981) who reported increased tissue concentrations of $PGF_{2\alpha}$ in guinea-pig placenta from day 30 of pregnancy that remained unchanged during the second half of gestation. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from guinea-pig sub-placenta were also increased on day 29 of pregnancy when compared to day 22, particularly in the first 2 h of culture. PG output from guinea-pig placenta was greater than PG output from the sub-placenta in culture, particularly $PGF_{2\alpha}$ output. These results essentially confirm the previous

study of Norman & Poyser (1998b). It has been suggested that the guinea-pig placenta has a decreased capacity for PG metabolism the final third of gestation (Moussard *et al.*, 1986). This study has shown that the PGFM output from the guinea-pig placenta was very high. This high output of PGFM could mean that the basal $PGF_{2\alpha}$ outputs from day 22 and day 29 guinea-pig placentae have been underestimated and that perhaps the metabolite levels should have been measured throughout along with PG outputs. When the outputs of $PGF_{2\alpha}$ from day 22 and day 29 are considered together with the PGFM outputs from day 22 and day 29 placenta, it does appear that there may be a decrease in metabolism within the first 2 h of culture. After 2 h of culture, metabolism does appear to increase with increase $PGF_{2\alpha}$ production by the day 29 guinea-pig placenta. The metabolism of $PGF_{2\alpha}$ may be an important mechanism for maintaining pregnancy and may act as a barrier to prevent primary active PGs getting to the myometrium and causing unwanted contraction of the mid-pregnant uterus. The present results have shown an increase in $PGF_{2\alpha}$ output from the guinea-pig placenta between day 22 and day 29 of pregnancy. Further investigation is required to determine whether metabolism does actually decrease as pregnancy proceeds. Rice *et al.* (1988) observed increased PG output from the sheep placenta in the final third of gestation and it has been suggested that this may be in preparation for parturition (Thorburn, 1991). This could also be the case for increased PG output from the guinea-pig placenta. However, further investigation is required as this study has only investigated PG outputs from day 29 of pregnancy (gestation normally 65-70 days).

Homogenisation of day 22 and 29 placenta gave an indication of PG synthesising capacity. Basal $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by day 29 placenta tended to increase compared to $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by day 22 guinea-pig placenta. This is consistent with the work of Moussard *et al.* (1986), who observed a further 3-fold increase in production rates of PGE_2 and $PGF_{2\alpha}$ by guinea-pig placental homogenates after day 50 of pregnancy. These results suggest that an increase in PGHS synthesis is responsible for increased $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by day 29 guinea-pig placenta. Schellenberg & Kirby (1997) demonstrated that the administration of exogenous arachidonic acid had no effect on PG output from guinea-pig placenta on days 45 to 65 of pregnancy, which indicates that there is a large pool of free endogenous arachidonic acid available for PG synthesis. This has also been observed in the human placenta (Ogburn *et al.*, 1988; Walsh, 1985) and suggests that the amount of PGHS is the rate limiting step for PG production by the placenta.

Indomethacin is a non-selective inhibitor of PGHS and NS-398 is selective for PGHS-2, but both have been shown to have similar potencies on PGHS-2 activity (Futaki *et al.*, 1993, 1994). After 22 days of gestation, $PGF_{2\alpha}$ output from the guinea-pig placenta was significantly reduced by indomethacin and NS-398. There was very little difference between treatment with indomethacin and NS-398 suggesting that PGHS-2 is the predominant enzyme involved in $PGF_{2\alpha}$ synthesis from day 22 guinea-pig placenta. Indomethacin (5 and 20 μM) and NS-398 (5 and 20 μM) were equipotent at inhibiting $PGF_{2\alpha}$ output from day 29 guinea-pig placenta which indicates that PGHS-2 is the predominant enzyme in the synthesis of $PGF_{2\alpha}$ from day

29 guinea-pig placenta. However, at a higher concentration of 40 μM, indomethacin was significantly greater at inhibiting $PGF_{2\alpha}$ output from day 29 placenta suggesting that both PGHS-1 and PGHS-2 are controlling $PGF_{2\alpha}$ synthesis. These results suggest that although PGHS-2 is the predominant enzyme responsible for $PGF_{2\alpha}$ synthesis in the guinea-pig placenta after 22 days of pregnancy, the increase in $PGF_{2\alpha}$ output at day 29 may also involve PGHS-1. Although PGE_2 output from day 22 guinea-pig placenta was significantly inhibited by both indomethacin and NS-398, treatment with indomethacin was significantly better. Indomethacin and NS-398 at low concentrations (5 and 20 μM) showed very little effect on PGE_2 output from day 29 guinea-pig placenta. At a higher concentration (40 μM), both indomethacin and NS-398 significantly reduced PGE_2 output. PGE_2 output from day 29 placenta in the presence of indomethacin was significantly lower than in the presence of NS-398. This suggests that both PGHS-1 and PGHS-2 enzymes are responsible for PGE_2 output from day 22 and day 29 guinea-pig placenta. After 22 days of gestation, 6-keto-$PGF_{1\alpha}$ output from guinea-pig placenta was inhibited slightly more by indomethacin than by NS-398, possibly indicating that both PGHS enzymes are involoved in 6-keto-$PGF_{1\alpha}$ synthesis. 6-Keto-$PGF_{1\alpha}$ output from day 29 guinea-pig placenta was inhibited by indomethacin and NS-398 throughout the 24 h culture period, and was significantly lower in the presence of indomethacin compared to NS-398 treatment. Therefore, both PGHS-1 and PGHS-2 appear to be responsible for 6-keto-$PGF_{1\alpha}$ synthesis from day 29 guinea-pig placenta as well as from day 22 placenta. As NS-398 has no significant inhibitory effect on PGHS-1 (Futaki *et al.*, 1993, 1994; Masferrer *et al.*, 1994) and indomethacin has a more potent action on

PGHS-1 than on PGHS-2 (Mitchell *et al.*, 1993), it appears that PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis from day 29 guinea-pig placenta is controlled by both PGHS enzymes.

$PGF_{2\alpha}$ and PGE_2 outputs from guinea-pig sub-placenta after 29 days of pregnancy were inhibited by indomethacin and NS-398. Indomethacin appeared to be more effective at inhibiting $PGF_{2\alpha}$ and PGE_2 outputs from the sub-placenta, which suggests that both forms of the PGHS enzyme are responsible for their outputs. 6-Keto-$PGF_{1\alpha}$ output from day 29 guinea-pig sub-placenta was inhibited throughout culture by indomethacin and NS-398. At smaller concentrations (5 and 20 μM), indomethacin appeared to be more effective at inhibiting 6-keto-$PGF_{1\alpha}$ output but, at 40 μM, indomethacin and NS-398 were equipotent. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 22 guinea-pig sub-placenta were significantly reduced throughout the 24 h culture period by both indomethacin and NS-398. All PGs measured were inhibited to a greater extent by indomethacin compared to treatment with NS-398. After 22 days of gestation $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from guinea-pig sub-placenta appear to be controlled by both forms of the PGHS enzyme and, after 29 days of pregnancy, this does not appear to change.

Previous studies have provided evidence which suggests that PGHS activity in the guinea-pig, sheep and pig placenta gradually increases during pregnancy (Moussard *et al.*, 1986; Rice *et al.*, 1988, 1989). The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by homogenates of day 22 placenta incubated for 1 h were reduced by indomethacin. Treatment of day 22 placenta homogenates with NS-398 showed no significant decrease in $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production although

production tended to decrease. $PGF_{2\alpha}$ and PGE_2 production was significantly higher in the presence of NS-398 when compared to treatment with indomethacin. The previous culture experiments suggested that PGHS-2 was the predominant enzyme responsible for $PGF_{2\alpha}$ production by day 22 guinea-pig placenta. However, homogenisation of this tissue indicates that both PGHS-1 and PGHS-2 are involved in $PGF_{2\alpha}$ production by day 22 placenta. Therefore, PG production by the day 22 guinea-pig placenta, cultured for 24 h, appears to be dependent on PGHS-2, while PG production by homogenates of day 22 placenta is dependent on both PGHS-1 and PGHS-2. The reason for this is not clear. Homogenisation of day 22 guinea-pig placenta suggest that PGHS-1 and PGHS-2 are responsible for PGE_2 and 6-keto-$PGF_{1\alpha}$ production. Indomethacin is a non-selective inhibitor of PGHS although it has previously been reported that it is more effective at inhibiting PGHS-1 than PGHS-2 (Mitchell *et al.*, 1993). NS-398 is a selective inhibitor of PGHS-2 (Futaki *et al.*, 1993, 1994). Both compounds were capable of inhibiting PGE_2 and 6-keto-$PGF_{1\alpha}$ production by homogenates of day 22 guinea-pig placenta, suggesting that PGHS-1 and PGHS-2 are responsible for PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis by this tissue.

Indomethacin and NS-398 significantly reduced $PGF_{2\alpha}$ and PGE_2 production by homogenates of day 29 guinea-pig placenta, while 6-keto-$PGF_{1\alpha}$ production was inhibited by indomethacin only. Indomethacin was significantly greater at inhibiting PGE_2 and 6-keto-$PGF_{1\alpha}$ production suggesting that both PGHS-1 and PGHS-2 are responsible for their production. Indomethacin and NS-398 were equipotent at inhibiting $PGF_{2\alpha}$ production by placental homogenates, which is consistent with the tissue culture experiments that suggested PGHS-2 is the predominant enzyme

responsible for $PGF_{2\alpha}$ production. These results suggest that both isoforms of the PGHS enzyme are responsible for PGE_2 and 6-keto-$PGF_{1\alpha}$ production by day 29 guinea-pig placenta and that PGHS-2 is the predominant enzyme involved in $PGF_{2\alpha}$ production by day 29 placenta.

Up-regulation of PGHS expression is a common effect of many agents that stimulate PG synthesis including growth factors, hormones, interleukins and phorbol esters (DeWitt, 1991), but no one signal transduction mechanism has been implicated in increased PGHS expression. However, activation of protein kinase C (PKC) has been observed to lead to increased PGHS expression in many cases including interleukin-1 (IL-1) stimulation of human dermal and endothelial cells (Raz *et al.*, 1988; Maier *et al.*, 1990). PKC has also been observed to stimulate mitogen-activated protein kinase (MAPK) (Qiu & Leslie, 1994) which stimulates PGHS-2 expression in macrophages (Hwang *et al.*, 1997).

In summary, this work has shown that PG output from guinea-pig placenta and sub-placenta increases between day 22 and 29 of pregnancy. Metabolism of $PGF_{2\alpha}$ by the guinea-pig placenta requires further investigation as it was not clear from this study whether metabolism decreases or increases between day 22 and day 29 of pregnancy. The finding that the PG synthesising capacity of placental homogenates increases between days 22 and 29 of pregnancy suggests that the increase in placental PG output between these two days was due to increased PGHS synthesis. It has been suggested that PGHS activity plays a key role in the maintenance of pregnancy (Kelly, 1994). PGHS is required for PG production. Previous workers have suggested that PGE_2 may be the important PG as pregnancy proceeds (see Thorburn, 1991).

PGE_2 output from the guinea-pig placenta and sub-placenta did not increase significantly between day 22 and day 29 of pregnancy. However, whether PGE_2 output from the guinea-pig placenta and sub-placenta increases after day 29 requires further investigation. At day 29, $PGF_{2\alpha}$ appears to be the important PG produced by the guinea-pig placenta and sub-placenta.

Previous workers have observed PGHS-2 to be the predominant enzyme in PG synthesis from the ovine and human placenta (Wimsatt *et al.*, 1993; Freed *et al.*, 1995; Rice *et al.*, 1995; Macchia *et al.*, 1997). This functional study showed that PGHS-2 is the predominant enzyme responsible for $PGF_{2\alpha}$ synthesis by day 22 and 29 guinea-pig placenta and sub-placenta, and that both isoforms of the PGHS enzyme are involved in PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis from day 22 and day 29 guinea-pig placenta and sub-placenta. PGHS-1 and PGHS-2 are often co-expressed in the same cell, and it has been proposed that PGHS-2 may co-localise with PGHS-1 to augment the function of PGHS-1 or substitute for it if PGHS-1 is lacking (Smith *et al.*, 1996). PGHS-2 is also associated with cell differentiation and replication (Otto & Smith, 1995) which could be an advantage for the developing fetus and placenta. The signal transduction mechanism responsible for stimulation of PGHS expression and increased PG synthesis from the guinea-pig placenta and sub-placenta as pregnancy proceeds requires further investigation.

3B.2 PROTEIN SYNTHESIS INHIBITORS

Introduction:

While synthesis of prostaglandins is regulated acutely by the activation of phospholipases and release of arachidonic acid from membrane phospholipids, net prostanoid production is dependent on the level of expression of prostaglandin H synthase (PGHS). Prostaglandin production by the guinea-pig placenta and sub-placenta has been observed to increase as pregnancy proceeds (see Section 3B.1.1). This increase has been attributed to stimulation of PGHS synthesis, which is dependent on the synthesis of proteins. PGHS is destroyed during PG biosynthesis, therefore, fresh enzyme must be provided if biosynthesis is to be maintained (Lands *et al.*, 1973). Continued protein synthesis is necessary for PGHS and PG synthesis in both cell types of guinea-pig endometrium (Naderali & Poyser, 1996b). Protein synthesis inhibitors have previously been observed to inhibit the output of PGs, particularly of $PGF_{2\alpha}$ from guinea-pig endometrium on days 7 and 15 of the oestrous cycle (Riley & Poyser, 1989). In addition, the protein synthesis inhibitors prevented the synthesis of PGHS in the guinea-pig endometrium during culture (Riley & Poyser, 1989).

Three different protein synthesis inhibitors with different mechanisms of action were chosen. The effects of actinomycin D (a DNA-dependent RNA synthesis inhibitor), puromycin (a releaser of nascent polypeptide chains before their synthesis is complete) and cycloheximide (an inhibitor of elongation step of transcription) on PG production by day 29 guinea-pig placenta and sub-placenta have been investigated.

Methods:

The placentae from day 29 pregnant guinea-pigs were removed, separated and prepared as described in section 2.1.4. The placenta and the sub-placenta were cultured separately in Petri dishes containing TCM plus cycloheximide (5, 10, 100 and 200 μM), puromycin (5, 10, 100 and 200 μM) or actinomycin D (10, 20, 50 and 100 μM). Dishes were placed in Kilner jars, gassed and incubated as described in Section 2.1.4. The TCM was removed and replaced after 2, 8 and 24 h. Samples were stored at -20°C and assayed for their $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ content. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Results:

Experiment 3B.2.1 The Effects of Cycloheximide, Puromycin and Actinomycin D on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

Low Concentrations of Protein Synthesis Inhibitors

$PGF_{2\alpha}$ output was unaffected by cycloheximide (5 and 10 μM) and puromycin (5 and 10 μM) after 2 and 8 h of culture (Fig. 3B.2.1.1a). After 24 h of culture, cycloheximide (10 μM, but not 5 μM) and puromycin (5 μM, but not 10 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output (Fig. 3B.2.1.1a). After 2 and 8 h

of culture, $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) inhibited by actinomycin D (10 and 20 μM), but not after 24 h of culture (Fig. 3B.2.1.1a). None of the protein synthesis inhibitors used had any significant effect on PGE_2 output from the placenta throughout the 24 h culture period (Fig. 3B.2.1.1b). 6-Keto-$PGF_{1\alpha}$ output was unaffected by cycloheximide (5 and 10 μM), puromycin (5 and 10 μM) and actinomycin D (10 and 20 μM) after 2 h of culture (Fig. 3B.2.1.1c). 6-Keto-$PGF_{1\alpha}$ output was reduced significantly ($P < 0.05$, n=5) by cycloheximide (5 and 10 μM) after 8 h and 24 h of culture (Fig. 3B.2.1.1c). Puromycin (5 and 10 μM) significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 8 h and 24 h, except 5 μM puromycin after 8 h of culture (Fig. 3B.2.1.1c). Actinomycin D (10 and 20 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output after 8 h. After 24 h of culture actinomycin D (20 μM, but not 10 μM) significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output (Fig. 3B.2.1.1c).

High Concentrations of Protein Synthesis Inhibitors

Cycloheximide (200 μM, but not 100 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output from the guinea-pig placenta after 2 and 8 h of culture, but had no effect after 24 h (Fig. 3B.2.1.3a). $PGF_{2\alpha}$ output was increased significantly ($P < 0.05$, n=5) by cycloheximide (100 μM) after 8 h and 24 h of culture, but had no effect after 2 h of culture (Fig. 3B.2.1.3a). Puromycin (100 and 200 μM) and actinomycin D (50 and 100 μM) had no effect on $PGF_{2\alpha}$ output except, after 2 h of culture, 200 μM puromycin and 100 μM actinomycin D significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output (Fig. 3B.2.1.3a). PGE_2 output was significantly ($P < 0.05$, n=5) reduced by

cycloheximide (100 and 200 μM) after 2 and 8 h of culture, and by cycloheximide (100 μM, but not 200 μM) after 24 h of culture (Fig. 3B.2.1.3b). Puromycin (100 μM, but not 200 μM) and actinomycin D (50 μM, but not 100 μM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output after 2 and 8 h of culture, but had no effect after 24 h of culture (Fig. 3B.2.1.3b). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) inhibited by cycloheximide (100 and 200 μM) and actinomycin D (50 μM, but not 100 μM) after 2 and 8 h of culture, but had no significant effect after 24 h (Fig. 3B.2.1.3c). After 24 h of culture, but not after 2 and 8 h of culture, cycloheximide (200 μM, but not 100 μM) significantly ($P < 0.05$, n=5) increased 6-keto-$PGF_{1\alpha}$ output (Fig. 3B.2.1.3c). Puromycin (100 and 200 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output throughout the culture period, except 100 μM puromycin which significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 8 h of culture (Fig. 3B.2.1.3c).

Sub-Placenta

Low Concentrations of Protein Synthesis Inhibitors

Cycloheximide (5 and 10 μM) had no significant effect on $PGF_{2\alpha}$ output from the sub-placenta after 2 and 8 h of culture (Fig. 3B.2.1.2a). After 24 h of culture cycloheximide (5 and 10 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output (Fig. 3B.2.1.2a). Puromycin (5 μM, but not 10 μM) and actinomycin D (10 μM, but not 20 μM) significantly reduced $PGF_{2\alpha}$ output from the guinea-pig sub-placenta after 2 h of culture (Fig. 3B.2.1.2a). $PGF_{2\alpha}$ output was increased significantly ($P < 0.05$, n=5) by puromycin (5 and 10 μM) after 8 h and 24 h of culture, and by actinomycin D (10 and 20 μM) after 24 h, but not after 8 h of culture (Fig.

3B.2.1.2a). Cycloheximide (5 μM, but not 10 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output after 2 and 8 h of culture, but not after 24 h (Fig. 3B.2.1.2b). After 24 h, PGE_2 output was increased significantly ($P < 0.05$, n=5) by cycloheximide (10 μM, but not 5 μM) and puromycin (10 μM, but not 5 μM) (Fig. 3B.2.1.2b). Puromycin (5 and 10 μM) had no effect on PGE_2 output after 2 h and 8 h of culture (Fig. 3B.2.1.2b). Actinomycin D (10 and 20 μM) reduced PGE_2 output significantly ($P < 0.05$, n=5) after 2 and 8 h of culture, except 10 μM actinomycin D which had no effect on PGE_2 output after 8 h (Fig. 3B.2.1.2b). Actinomycin D (10 and 20 μM) had no effect on PGE_2 output after 24 h of culture (Fig. 3B.2.1.2b). None of the protein synthesis inhibitors used had any significant effect on 6-keto-$PGF_{1\alpha}$ output from the guinea-pig sub-placenta throughout the 24 h culture period (Fig. 3B.2.1.2c).

High Concentrations of Protein Synthesis Inhibitors

$PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) inhibited after 2 h and 24 h of culture by cycloheximide (100 μM, but not 200 μM), and after 8 h by cycloheximide (100 and 200 μM) (Fig. 3B.2.1.4a). After 24 h of culture, but not after 2 and 8 h of culture, cycloheximide (200 μM, but not 100 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output from the sub-placenta (Fig. 3B.2.1.4a). Puromycin (100 and 200 μM) significantly ($P < 0.05$, n=5) decreased $PGF_{2\alpha}$ output from the sub-placenta after 2 and 8 h of culture, but had no significant effect after 24 h (Fig. 3B.2.1.4a). Actinomycin D (50 μM, but not 100 μM) significantly ($P < 0.05$, n=5) decreased $PGF_{2\alpha}$ output after 8 h of culture, but not after 2 and 24 h (Fig. 3B.2.1.4a).

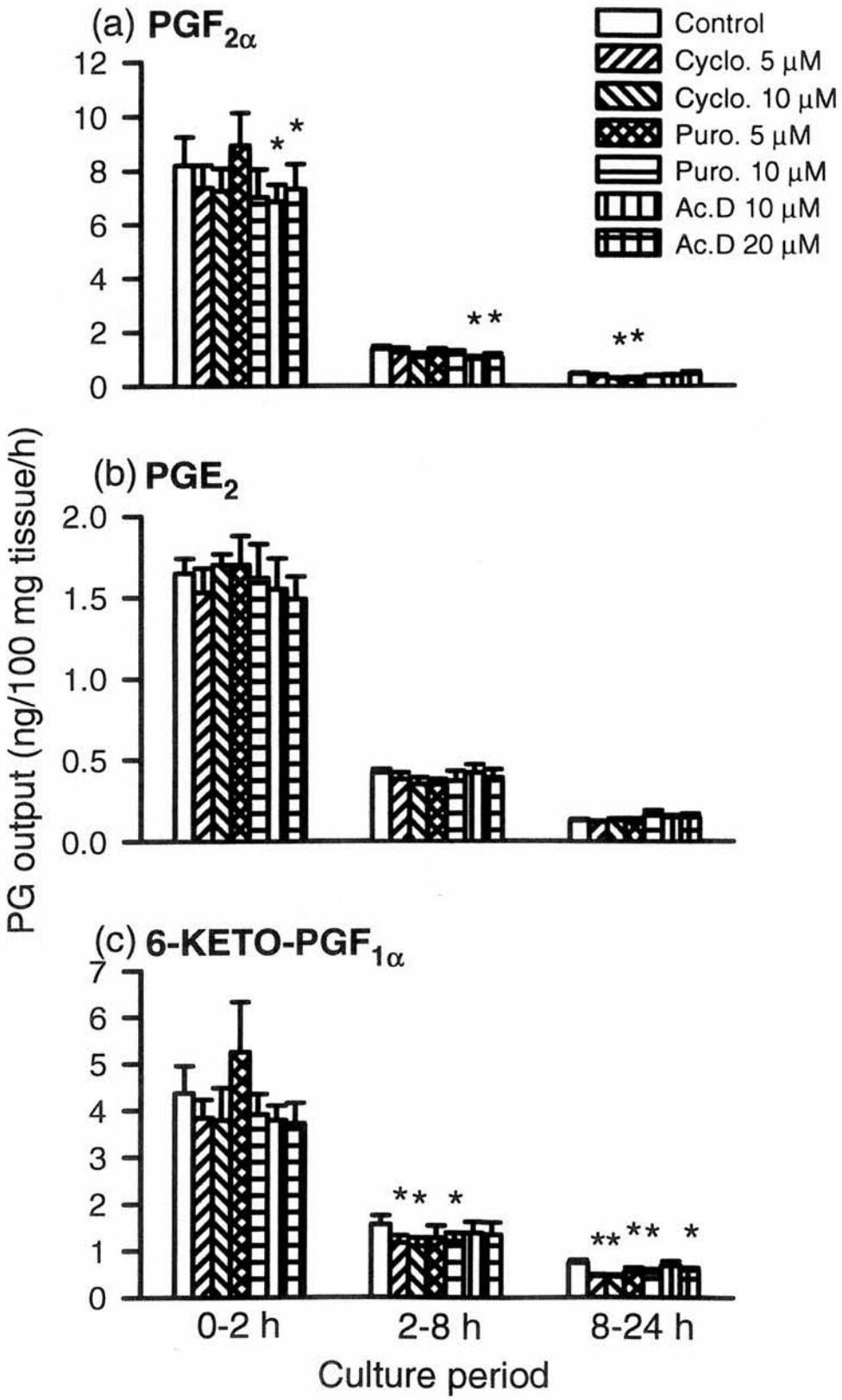


Figure 3B.2.1.1 Effects of cycloheximide (5 and 10 μM), puromycin (5 and 10 μM) and actinomycin D (10 and 20 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.

*Significantly lower, $P < 0.05$, than corresponding control value.

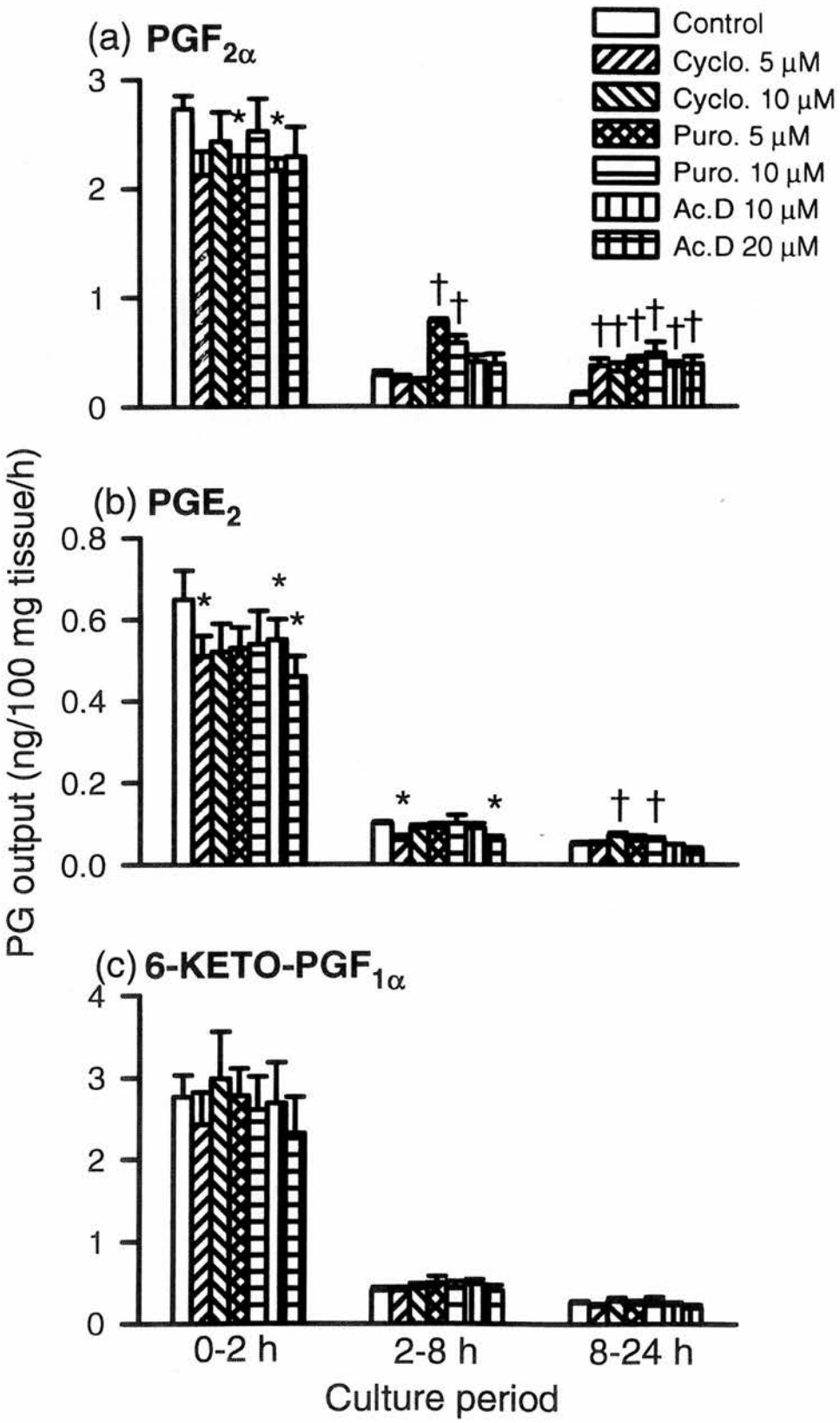


Figure 3B.2.1.2 Effects of cycloheximide (5 and 10 μM), puromycin (5 and 10 μM) and actinomycin D (10 and 20 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.
*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

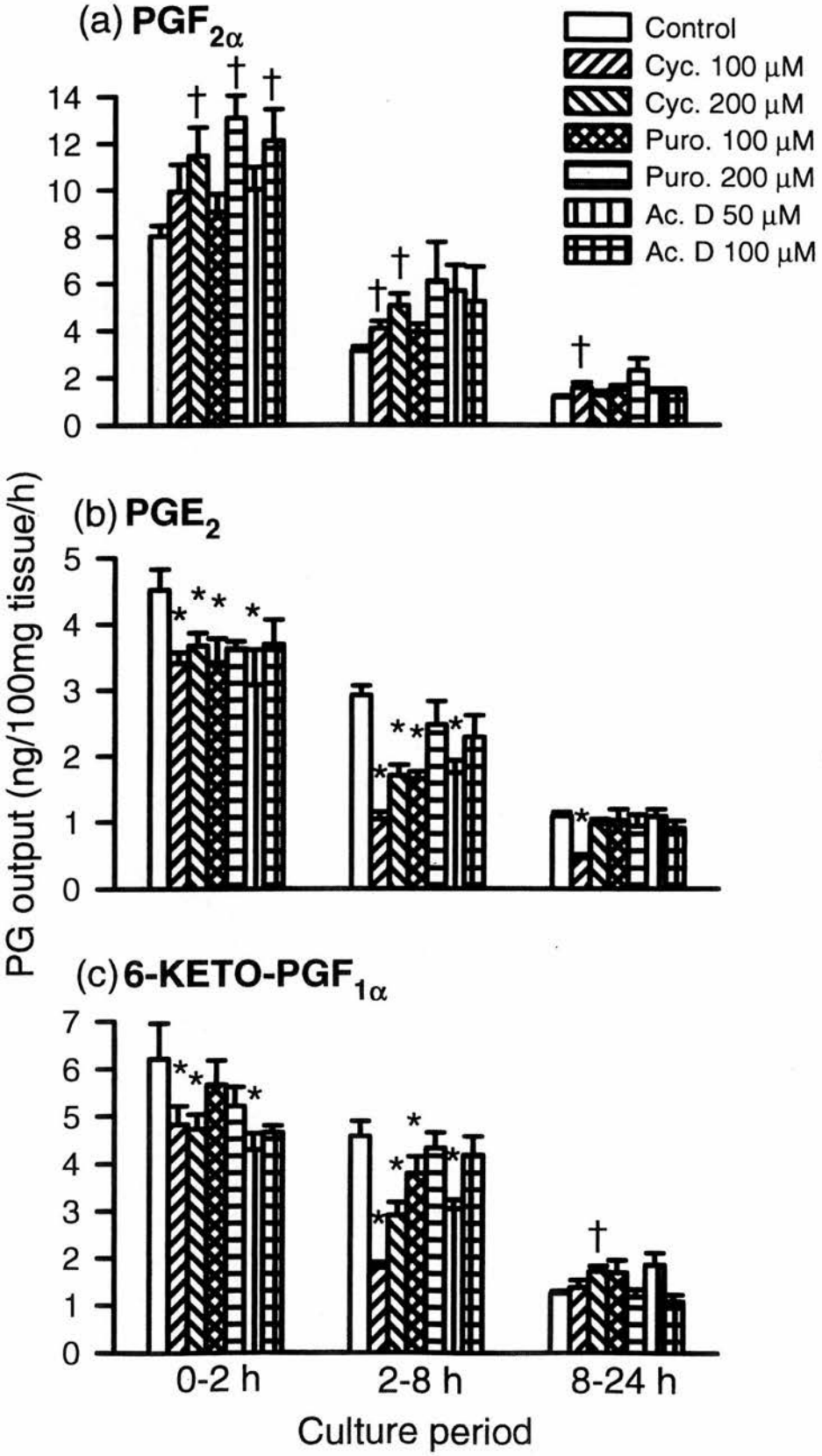


Figure 3B.2.1.3 Effects of cycloheximide (100 and 200 μM), puromycin (100 and 200 μM) and actinomycin D (50 and 100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.
*/† Significantly lower/higher, $P < 0.05$, than corresponding control value.

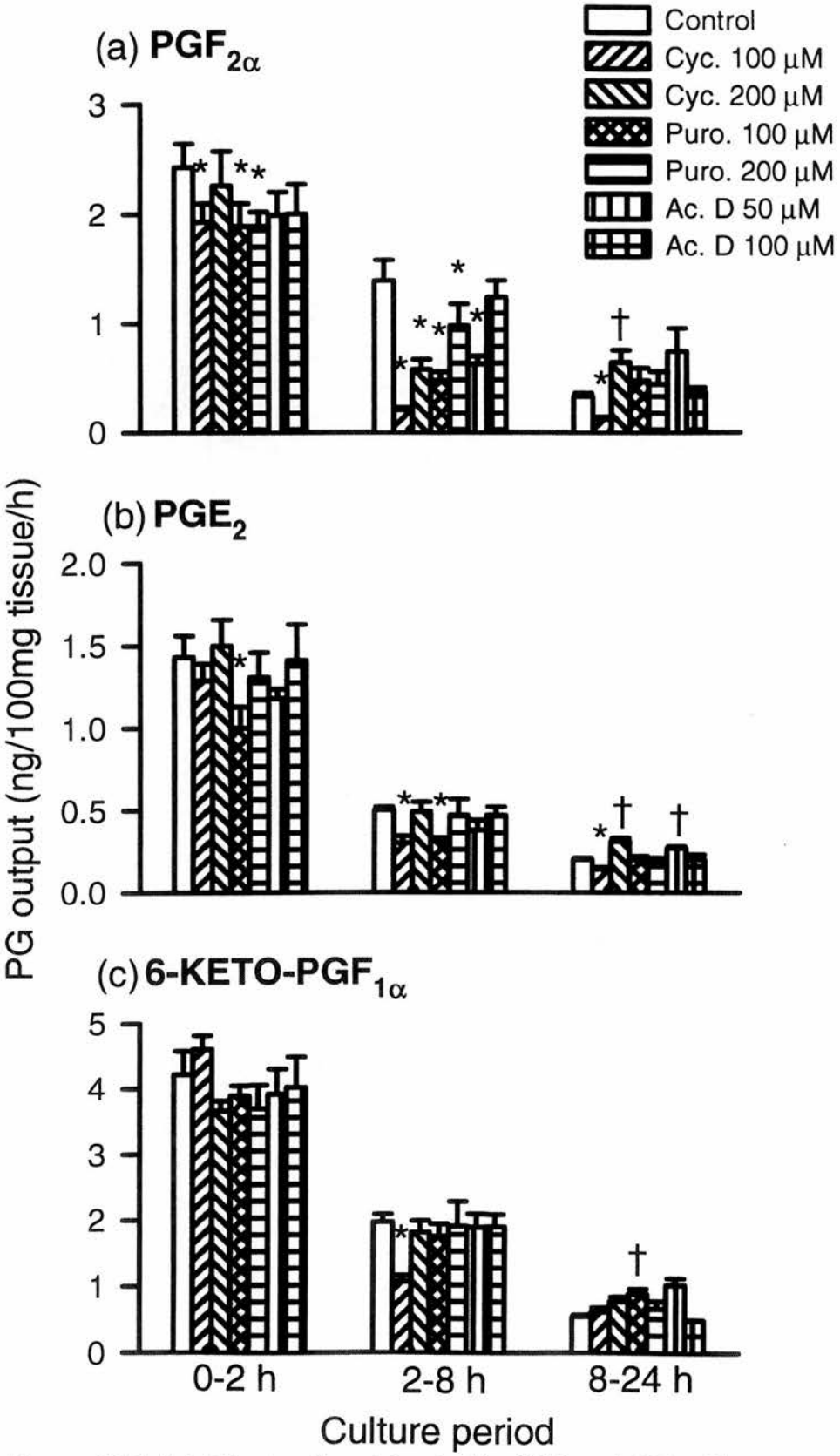


Figure 3B.2.1.4 Effects of cycloheximide (100 and 200 μM), puromycin (100 and 200 μM) and actinomycin D (50 and 100 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$,(b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.
*/† Significantly lower/higher, $P < 0.05$, than corresponding control value.

Cycloheximide (100 and 200 μM) had no effect on PGE_2 output after 2 h of culture, but after 8 h and 24 h of culture, cycloheximide (100 μM, but not 200 μM) reduced PGE_2 output significantly ($P < 0.05$, n=5) (Fig. 3B.2.1.4b). After 24 h, but not after 2 and 8 h of culture, cycloheximide (200 μM, but not 100 μM) significantly ($P < 0.05$, n=5) stimulated PGE_2 output (Fig. 3B.2.1.4b). Puromycin (100 μM, but not 200 μM) significantly ($P < 0.05$, n=5) decreased PGE_2 output after 2 and 8 h of culture, but had no effect after 24 h of culture (Fig. 3B.2.1.4b). PGE_2 output was unaffected by actinomycin D (50 and 100 μM) throughout the 24 h culture period, except after 24 h of culture, PGE_2 output was significantly ($P < 0.05$, n=5) increased by 50 μM actinomycin D (Fig. 3B.2.1.4b). 6-Keto-$PGF_{1\alpha}$ output was unaffected by the protein synthesis inhibitors used throughout culture, except by 100 μM cycloheximide which significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 8 h, and by 100 μM puromycin which significantly ($P < 0.05$, n=5) increased 6-keto-$PGF_{1\alpha}$ output after 24 h of culture (Fig. 3B.2.1.4c).

Discussion:

Cycloheximide and puromycin at low concentrations of 10 μM and 5 μM, respectively, inhibited $PGF_{2\alpha}$ output from day 29 guinea-pig placenta after 24 of culture. Actinomycin D at low concentrations also inhibited $PGF_{2\alpha}$ output from the guinea-pig placenta after 2 h and 8 h of culture. These results suggest that $PGF_{2\alpha}$ production by day 29 guinea-pig placenta may be dependent on protein synthesis. The effects of cycloheximide, puromycin and actinomycin D were not pronounced and this may have been due to the low concentrations used. However, at

higher concentrations, cycloheximide, puromycin and actinomycin D stimulated $PGF_{2\alpha}$ output from the guinea-pig placenta, particularly cycloheximide. Riley & Poyser (1989) have previously observed that protein synthesis inhibitors stimulate PG output from day 7 and day 15 superfused guinea-pig uterus, with cycloheximide having a particularly strong stimulatory effect on PGE_2 output. It has also been shown that PGHS mRNA can be superinduced by cycloheximide (DeWitt *et al.*, 1990; Maier *et al.*, 1990). This stimulation of PGHS, the enzyme responsible for the conversion of arachidonic acid to PGs, may account for the observed increase in $PGF_{2\alpha}$ output from day 29 guinea-pig placenta in the presence of cycloheximide.

PGE_2 output from the guinea-pig placenta was unaffected by the lower concentrations of cycloheximide, puromycin and actinomycin D used. Higher concentrations of these treatments inhibited PGE_2 output significantly, especially during the first 8 h of culture. This suggests that PGE_2 output from the guinea-pig placenta is dependent on the synthesis of proteins. The presence of protein synthesis inhibitors at low concentrations significantly inhibited 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta, especially after 24 h of culture. Higher concentrations of cycloheximide, puromycin and actinomycin D also inhibited 6-keto-$PGF_{1\alpha}$ output from the placenta, but had a more pronounced effect at the beginning of the culture period. These results show that both low and high concentrations of cycloheximide, puromycin and actinomycin D were capable of inhibiting 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta, and suggest that 6-keto-$PGF_{1\alpha}$ production by the placenta requires protein synthesis. After 24 h of culture, 6-keto-$PGF_{1\alpha}$ output was significantly increased by the presence of cycloheximide (200 μM). Naderali &

Poyser (1996b) observed that cycloheximide was not as effective at inhibiting PG output from cultured guinea-pig endometrium as actinomycin D and puromycin because it had a short-term stimulatory effect. A similar action may account for the effect of cycloheximide on the placenta.

Low concentrations of puromycin and actinomycin D inhibited $PGF_{2\alpha}$ output from day 29 guinea-pig sub-placenta after 2 h of culture. However, after 24 h of culture, all treatments used significantly stimulated $PGF_{2\alpha}$ output from the sub-placenta. This effect was not so pronounced when higher concentrations of these compounds were used. At these higher concentrations, cycloheximide, puromycin and actinomycin D inhibited $PGF_{2\alpha}$ output from the sub-placenta after 8 h of culture, while only cycloheximide significantly increased $PGF_{2\alpha}$ output after 24 h of culture. High concentrations of puromycin and actinomycin D had no significant stimulatory effect on $PGF_{2\alpha}$ output from the guinea-pig sub-placenta after 24 h of culture. These results suggest that $PGF_{2\alpha}$ production by the sub-placenta may be dependent on protein synthesis. Cycloheximide and actinomycin D (low concentrations) significantly inhibited PGE_2 output from the sub-placenta after 2 and 8 h of culture, but had little effect at higher concentrations. Puromycin had no inhibitory effect on PGE_2 output at low concentrations of 5 and 10 μM, but significantly reduced PGE_2 output after 2 and 8 h of culture at a higher concentration of 100 μM. The inhibitory effects of cycloheximide, puromycin and actinomycin D on PGE_2 output from the sub-placenta suggests that protein synthesis is necessary for PGE_2 production. PGE_2 output from the sub-placenta was stimulated after 24 h of culture in the presence of cycloheximide at both high and low concentrations, puromycin at a low

concentration and by actinomycin D at a high concentration. Cycloheximide has previously been observed to stimulate PGE_2 output from the superfused guinea-pig uterus (Riley & Poyser, 1989) and is known to induced PGHS mRNA (DeWitt *et al.*, 1990; Maier *et al.*, 1990). 6-Keto-$PGF_{1\alpha}$ output from day 29 sub-placenta was unaffected by the presence of low concentrations of cycloheximide, puromycin and actinomycin D. However, at higher concentrations, cycloheximide was observed to inhibit 6-keto-$PGF_{1\alpha}$ output after 8 h of culture, while puromycin was observed to stimulate 6-keto-$PGF_{1\alpha}$ output after 24 h of culture. It remains unclear whether 6-keto-$PGF_{1\alpha}$ production by the guinea-pig sub-placenta is dependent on the production of proteins. The stimulatory effects of the compounds used, previously observed when measuring $PGF_{2\alpha}$ and PGE_2 outputs from the guinea-pig placenta and sub-placenta, may explain why no inhibitory effect on 6-keto-$PGF_{1\alpha}$ output from the sub-placenta was seen. Cycloheximide, puromycin and actinomycin D may have opposing action with regards to PG production by the guinea-pig placenta and sub-placenta.

In summary, these results suggest that PG production by the day 29 guinea-pig placenta and sub-placenta may be dependent to a limited extent on the synthesis of proteins. PGHS is destroyed during PG biosynthesis, therefore fresh enzyme must be provided if biosynthesis is to be maintained (Lands *et al.*, 1973). Therefore, the process of protein synthesis may be necessary for the production of PGHS, the enzyme responsible for the conversion of arachidonic acid to PGs.

3B.3 AN INVESTIGATION INTO THE ROLE OF CALCIUM IN PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG PLACENTA.

Introduction:

'Phospholipase A_2' consists of a family of enzymes, some of which are dependent on calcium. Phospholipase A_2 is the enzyme responsible for arachidonic acid release from membrane phospholipids, and it is well documented that arachidonic acid release for PG synthesis is triggered in cells by many calcium mobilising agonists (Clark *et al.*, 1995). PG production by the guinea-pig placenta and sub-placenta has been observed to increase as pregnancy proceeds (see Section 3B.1.1). The calcium (Ca^{2+}) requirement for this increased PG synthesis by guinea-pig placenta and sub-placenta has been investigated. Day 29 guinea-pig placenta and sub-placenta were cultured in calcium-depleted medium to determine if extracellular calcium is required for prostaglandin production.

It has previously been observed that raising the intracellular free calcium concentration stimulates $PGF_{2\alpha}$ synthesis by, and release from, the guinea-pig uterus (Poyser & Brydon, 1983). The effect of 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8; an intracellular Ca^{2+} antagonist) on PG production by the day 29 guinea-pig placenta and sub-placenta have been examined. This was to establish whether or not intracellular calcium is required for PG synthesis by these tissues. The effect of EGTA (a calcium chelator) on PG production by the day 29 guinea-pig placenta and sub-placenta were also studied. Riley & Poyser (1987b) have observed that EGTA, when cultured with the guinea-pig endometrium, acts intracellularly as well as extracellularly.

Calmodulin is a cytosolic calcium-binding protein through which the action of calcium on various enzymes is mediated. Calmodulin functions in a variety of cells (Cheung, 1980) and, in some tissues, PLA_2 is a calmodulin-dependent enzyme (Wong & Cheung 1979, Maskowitz *et al.*, 1983). Calmodulin has previously been observed to play a role in PG synthesis in the guinea-pig uterus (Poyser, 1985a, b; Riley & Poyser, 1987b). Day 29 guinea-pig placenta and sub-placenta were cultured for 24 h in the presence of trifluoperazine (TFP; a calmodulin antagonist) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7; a calmodulin antagonist) and PG output was observed. This experiment was carried out to determine whether calcium exerts its effect on the guinea-pig placenta and sub-placenta by combining with the intracellular calcium binding protein, calmodulin.

It is well documented that phorbol esters stimulate the activation of protein kinase C (PKC) in a number of tissues. Protein kinase C has been observed to increase arachidonic acid release in, and $PGF_{2\alpha}$ and PGE_2 production by human decidual cells (Schrey & Read, 1986). Phorbol esters also stimulate PGHS-2 mRNA expression in cultured human amnion cells (Zakar *et al.*, 1996). The effect of phorbol 12-myristate 13-acetate (TPA; a stimulator of protein kinase C) on PG output from day 29 guinea-pig placenta and sub-placenta was investigated. TPA mimics the action of diacylglycerol, thereby activating PKC. Anteby *et al.* (1997) have observed that PGHS-2 expression was increased in the presence of TPA, as was PGE_2 output, from human trophoblasts.

The effects of nifedipine and verapamil (voltage-dependent Ca^{2+} channel blockers) on PG outputs from day 29 guinea-pig placenta and sub-placenta were also

investigated. This was to determine whether any influx of calcium from outside the cells was via voltage-dependent channels.

The effects of EGTA, TMB-8, nifedipine and verapamil were also investigated in homogenates of day 29 guinea-pig placenta and sub-placenta to observe whether these treatments had any effect on the PG synthesising capacity.

Methods:

Day 29 guinea-pig placentae were removed and prepared as described in Section 2.1.4. In experiment 3B.3.1, the day 29 placenta and sub-placenta were cultured for 24 h in Medium 199 or in calcium-depleted Medium 199. These dishes were placed in modified Kilner jars, gassed and incubated as previously described (Section 2.1.4). The culture medium in each dish was changed and collected after 2, 8 and 24 h. Samples were stored at -20°C before being assayed for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ content. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

In experiments 3B.3.2-4, the placentae from day 29 pregnant guinea-pigs were removed and separated as described in Section 2.1.4. The placenta and sub-placenta were cut into small pieces and placed on lens tissue on top of raised gauze platforms in culture dishes containing tissue culture Medium 199 (TCM). In experiment 3B.3.2, day 29 guinea-pig placenta and sub-placenta were cultured separately in TCM containing EGTA (2 mM), TMB-8 (30 and 100 μM), TFP (100 and 200 μM) or W-7 (150 and 300 μM). Experiment 3B.3.3 was carried out similarly in the

presence of nifedipine (1, 10 and 100 μM) or verapamil (1, 10 and 100 μM). In experiment 3B.3.4, placenta and sub-placenta were cultured in the presence of TPA (1.5, 8 and 40 nM). In all three experiments dishes were placed in modified Kilner jars, and were gassed and incubated as previously described in Section 2.1.4. Culture medium was collected after 2, 8 and 24 h. Samples were stored at -20°C. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ content in each sample was measured using radioimmunoassay. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Homogenisation experiment 3B.3.5 was carried out using day 29 guinea-pig placenta. The tissue was blotted dry, weighed and then homogenised as described in Section 2.1.3 with Krebs solution containing either EGTA (2 mM), TMB-8 (30 and 100 μM), nifedipine (10 and 100 μM) or verapamil (10 and 100 μM). One sample in each experiment was untreated and acted as a control. Each sample was allowed to incubate for 60 min at 37°C (see Section 2.1.3), and prostaglandins were then extracted as described in Section 2.1.1. The ethyl acetate extracts were evaporated to dryness on a rotary evaporator, and the residues were each re-dissolved in 5 ml ethyl acetate and stored at -20°C until assayed for their PG content. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Results:

Experiment 3B.3.1 The Effect of Ca^{2+}-Depleted Medium on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

$PGF_{2\alpha}$ and PGE_2 outputs from guinea-pig placenta were unaffected by culturing the tissue in calcium-depleted medium for 24 h (Fig. 3B.3.1.1a and b). Calcium-depleted medium significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output from placenta throughout the 24 h culture period compared to culturing the placenta in Medium 199 (Fig. 3B.3.1.1c).

Sub-Placenta

Calcium-depleted medium significantly ($P < 0.05$, n=5) inhibited the $PGF_{2\alpha}$ output from the sub-placenta after 2 h of culture but, after 8 and 24 h, the presence of calcium-depleted medium had no significant effect (Fig. 3B.3.1.2a). PGE_2 output was reduced significantly ($P < 0.05$, n=5) throughout the 24 h culture period in the presence of calcium-depleted culture medium compared to culturing the tissue in Medium 199 (Fig. 3B.3.1.2b). 6-Keto-$PGF_{1\alpha}$ output was unaffected by calcium-depleted medium after 2 and 8 h of culture, but was significantly ($P < 0.05$, n=5) reduced by calcium-depleted medium after 24 h of culture (Fig. 3B.3.1.2c).

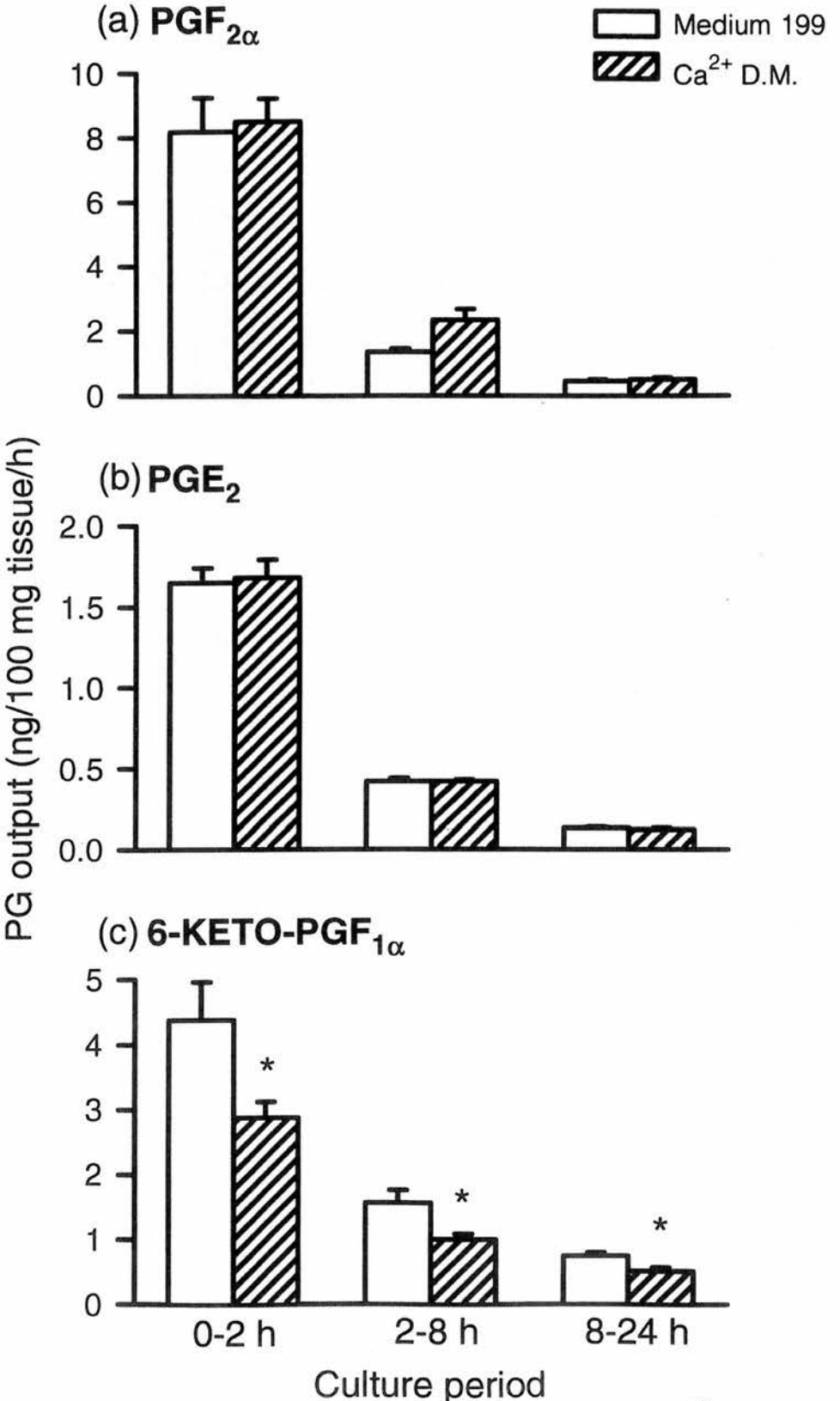


Figure 3B.3.1.1 Effect of calcium-depleted Medium (Ca^{2+} D.M.) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24h.
*Significantly, $P < 0.05$, lower than corresponding control value.

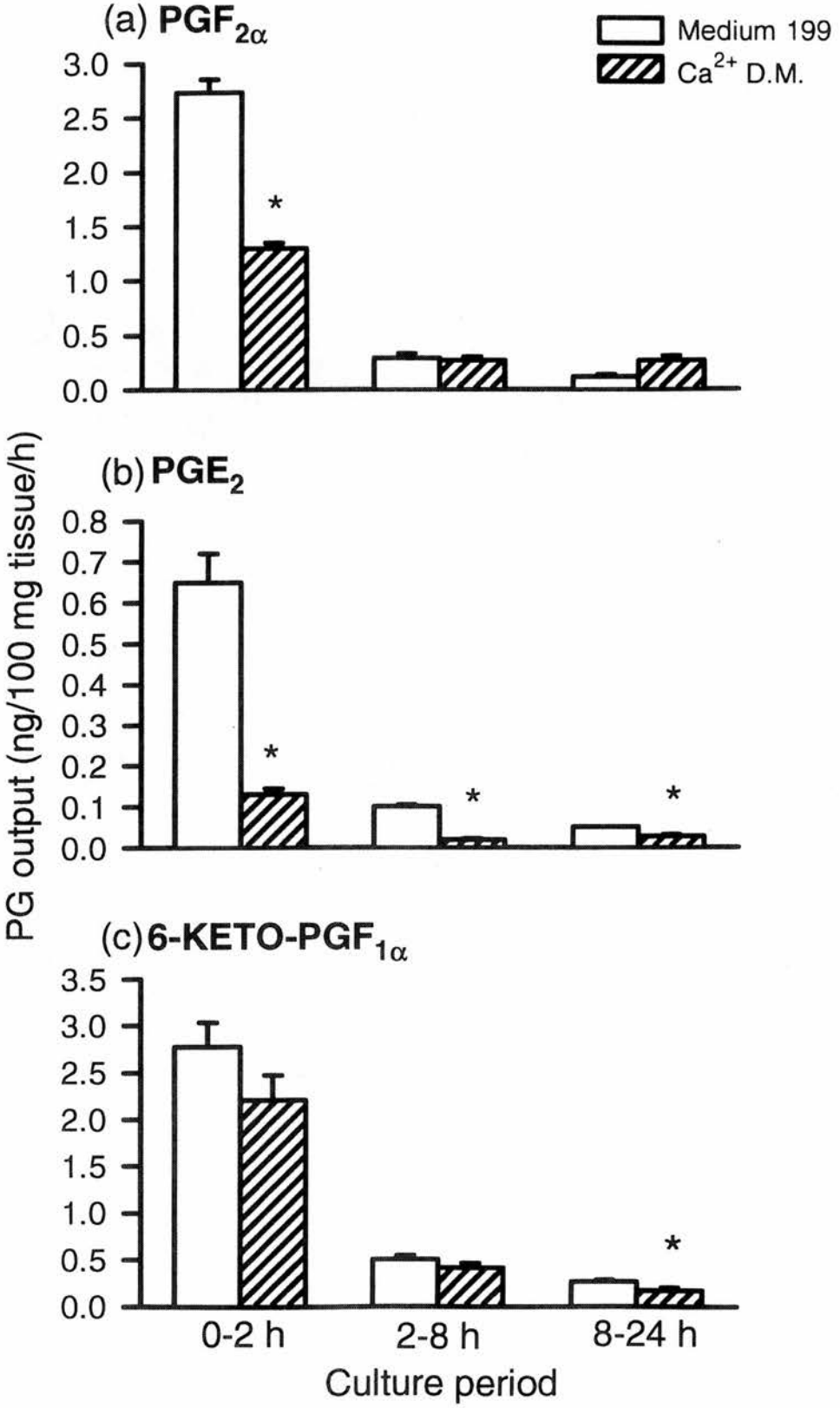


Figure 3B.3.1.2 Effect of calcium-depleted Medium (Ca^{2+} D.M.) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.

*Significantly, $P < 0.05$, lower than corresponding control value.

Experiment 3B.3.2 The Effects of a calcium chelator (EGTA), an intracellular antagonist (TMB-8) and calmodulin inhibitors (TFP and W-7) on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

$PGF_{2\alpha}$ output from the guinea-pig placenta was significantly ($P < 0.05$, n=5) decreased by EGTA (2 mM) after 8 and 24 h of culture, but EGTA (2 mM) had no effect on $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.2.1a). TMB-8 (30 and 100 μM) had no significant effect on $PGF_{2\alpha}$ output in the first 2 h of culture (Fig. 3B.3.2.1a). After 24 h of culture, but not after 8 h, TMB-8 (30 μM, but not 100 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output. After 8 and 24 h, TMB-8 (100 μM, but not 30 μM) stimulated $PGF_{2\alpha}$ ouput significantly ($P < 0.05$, n=5) (Fig. 3B.3.2.1a). TFP (100 and 200 μM) had no effect on $PGF_{2\alpha}$ output, except after 8 h, when 200 μM TFP significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output (Fig. 3B.3.2.1a). W-7 (150 and 300 μM) had no effect on $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.2.1a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) increased after 8 h by W-7 (150 and 300 μM), and after 24 h by W-7 (300 μM, but not 150 μM) (Fig. 3B.3.2.1a).

PGE_2 output from the placenta was unaffected by any of the treatments used, except TMB-8 (30 μM) which significantly ($P < 0.05$, n=5) reduced PGE_2 throughout the 24 h culture period (Fig. 3B.3.2.1b).

6-Keto-$PGF_{1\alpha}$ output was not affected by any of the treatments used after 2 h of culture (Fig. 3B.3.2.1c). 6-Keto-$PGF_{1\alpha}$ output was decreased significantly ($P < 0.05$,

n=5) by EGTA (2 mM) and TMB-8 (30 μM, but not 100 μM) after 8 and 24 h of culture (Fig. 3B.3.2.1c). TFP (100 μM, but not 200 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h of culture (Fig. 3B.3.2.1c). W-7 (150 and 300 μM) decreased 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 8 h of culture, but not after 24 h (Fig. 3B.3.2.1c).

Sub-Placenta

EGTA (2 mM) had no significant effect on $PGF_{2\alpha}$ output from the guinea-pig sub-placenta throughout the 24 h culture period (Fig. 3B.3.2.2a). TMB-8 (100 μM, but not 30 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.2.2a). After 8 and 24 h of culture, TMB-8 (30 μM, but not 100 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output from the sub-placenta (Fig. 3B.3.2.2a). $PGF_{2\alpha}$ output was increased significantly ($P < 0.05$, n=5) by TFP (200 μM, but not 100 μM) after 8 h and 24 h of culture, but had no effect after 2 h of culture (Fig. 3B.3.2.2a). W-7 (150 and 300 μM) had no effect on $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.2.2a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) increased by W-7 (300 μM, but not 150 μM) after 8 h, and by W-7 (150 and 300 μM) after 24 h of culture (Fig. 3B.3.2.2a and b).

PGE_2 output was significantly ($P < 0.05$, n=5) reduced after 2 h of culture by EGTA (2 mM) and TMB-8 (30 μM, but not 100 μM). PGE_2 output was not affected by EGTA or TMB-8 after 8 and 24 h of culture (Fig. 3B.3.2.2b). TFP (100 and 200 μM) had no significant effect on PGE_2 output after 2 and 8 h of culture (Fig. 3B.3.2.2b). After 24 h of culture TFP (100 and 200 μM) increased PGE_2 output significantly ($P <$

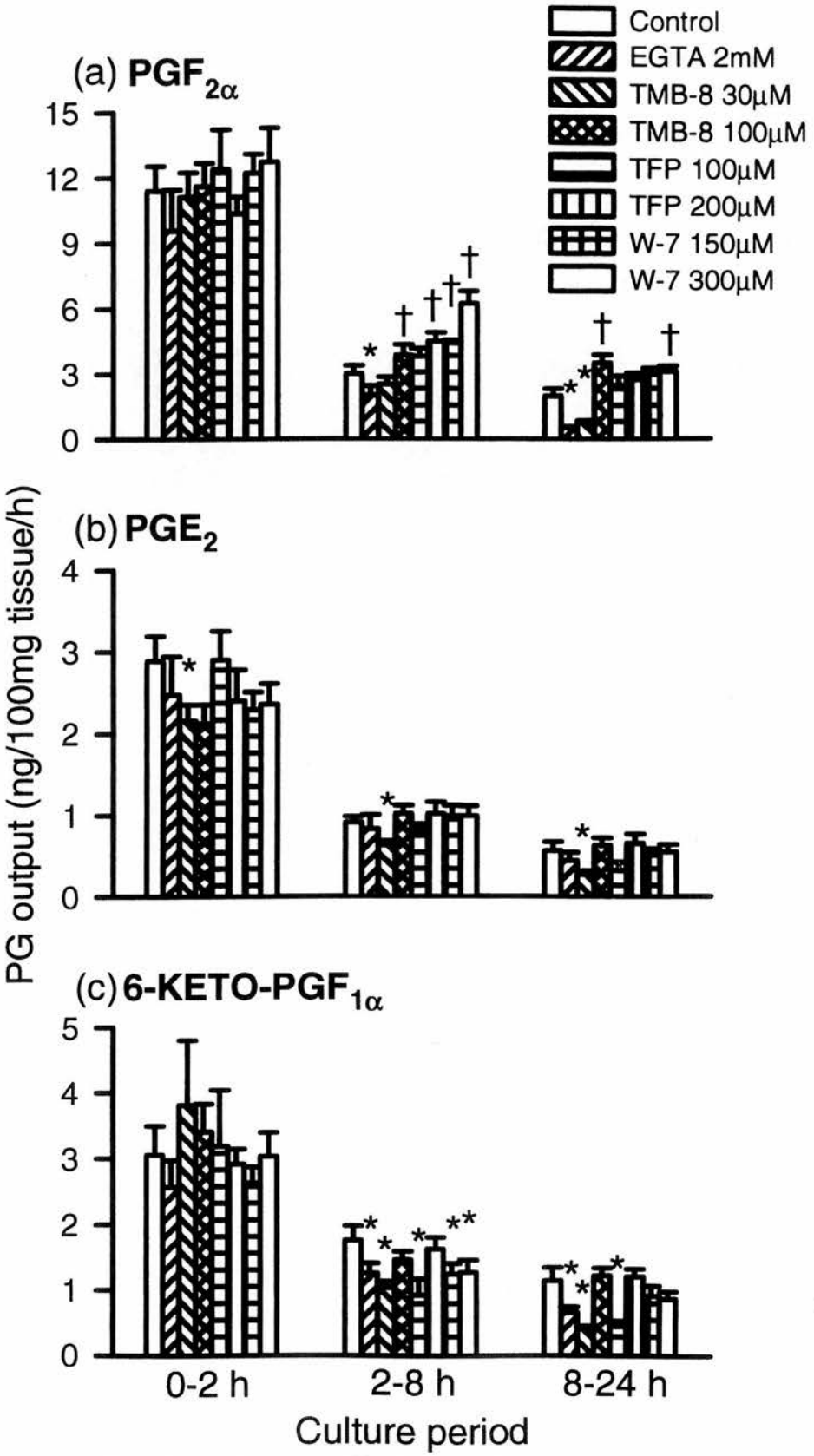


Figure 3B.3.2.1 Effects of EGTA (2 mM), TMB-8 (30 and 100 μM), TFP (100 and 200 μM) and W-7 (150 and 300 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.
*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

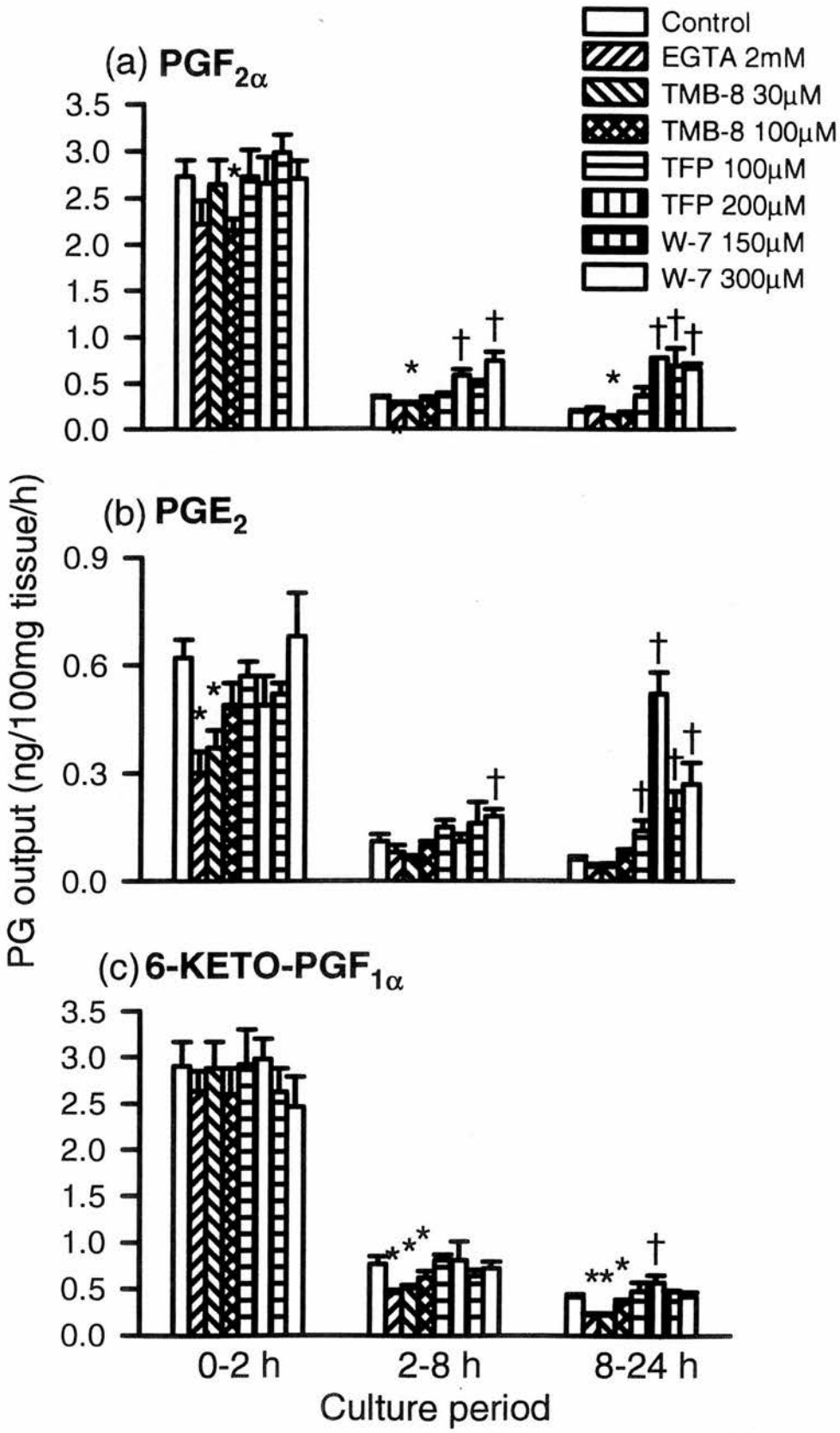


Figure 3B.3.2.2 Effects of EGTA (2 mM), TMB-8 (30 and 100μ M), TFP (100 and 200 μM) and W-7 (150 and 300 μM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.

*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

0.05, n=5) (Fig. 3B.3.2.2b). W-7 (150 and 300 μM) had no effect on PGE_2 output after 2 h of culture (Fig. 3B.3.2.2b). After 8 h of culture, W-7 (300 μM, but not 150 μM) significantly ($P < 0.05$, n=5) stimulated PGE_2 output, and after 24 h of culture W-7 (150 and 300 μM) increased PGE_2 output significantly ($P < 0.05$, n=5) (Fig. 3B.3.2.2b).

6-Keto-$PGF_{1\alpha}$ output was unaffected by EGTA (2 mM) and TMB-8 (30 and 100 μM) after 2 h of culture (Fig. 3B.3.2.2c). EGTA (2 mM) and TMB-8 (30 and 100 μM) reduced 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 8 h and 24 h of culture (Fig. 3B.3.2.2c). TFP (100 and 200 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output throughout the culture period, except TFP (200 μM) which significantly ($P < 0.05$, n=5) increased 6-keto-$PGF_{1\alpha}$ output after 24 h in culture (Fig. 3B.3.2.2c). W-7 (150 and 300 μM) had no significant effect on 6-keto-$PGF_{1\alpha}$ output from the guinea-pig sub-placenta throughout the 24 h culture period (Fig. 3B.3.2.2c).

Experiment 3B.3.3 The Effects of Calcium Channel Blockers (Nifedipine and Verapamil) on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

Nifedipine (10 and 100 μM, but not 1 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.3.1a). Nifedipine (1, 10 and 100 μM) significantly ($P < 0.05$, n=5) inhibited $PGF_{2\alpha}$ output after 8 and 24 h of culture (Fig.

3B.3.3.1a). Verapamil (1, 10 and 100 μM) had no significant effect on $PGF_{2\alpha}$ output from the guinea-pig placenta after 2 h of culture (Fig. 3B.3.3.1a). Verapamil (1 μM, but not 10 and 100 μM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output from the placenta after 8 and 24 h of culture, while verapamil (100 μM, but not 1 and 10 μM) increased output significantly ($P < 0.05$, n=5) after 24 h but had no significant effect after 8 h of culture (Fig. 3B.3.3.1a).

Nifedipine (1, 10 and 100 μM) had no significant effect on PGE_2 output from the guinea-pig placenta after 2 h of culture (Fig. 3B.3.3.1b). PGE_2 output was reduced significantly ($P < 0.05$, n=5) by nifedipine (10 and 100 μM, but not 1 μM) after 8 h of culture, and by nifedipine (1, 10 and 100 μM) after 24 h of culture (Fig. 3B.3.3.1b). Verapamil (1, 10 and 100 μM) had no significant effect on PGE_2 output after 2 and 8 h of culture, except 1 μM verapamil which significantly ($P < 0.05$, n=5) inhibited PGE_2 output after 2 h (Fig. 3B.3.3.1b). PGE_2 output was significantly ($P < 0.05$, n=5) inhibited by verapamil (1 μM, but not 10 and 100 μM), and significantly ($P < 0.05$, n=5) increased by verapamil (100 μM, but not 1 and 10 μM) after 24 h of culture (Fig. 3B.3.3.1b).

Nifedipine (1, 10 and 100 μM) had no significant effect on 6-keto-$PGF_{1\alpha}$ output after 2 h and 8 h of culture (Fig. 3B.3.3.1c). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) inhibited by nifedipine (1 and 10 μM, but not 100 μM) after 24 h of culture (Fig. 3B.3.3.1c). 6-Keto-$PGF_{1\alpha}$ output from the placenta was unaffected by verapamil (1, 10 and 100 μM) throughout the 24 h culture period, except after 24 h, 100 μM verapamil increased 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) (Fig. 3B.3.3.1c).

Sub-Placenta

$PGF_{2\alpha}$ output from the guinea-pig sub-placenta was unaffected by nifedipine (1, 10 and 100 μM) after 2 and 24 h of culture (Fig. 3B.3.3.2a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) reduced by nifedipine (1 and 100 μM, but not 10 μM) after 8 h of culture (Fig. 3B.3.3.2a). $PGF_{2\alpha}$ output was unaffected by verapamil (1, 10 and 100 μM) throughout the culture period, except for 100 μM verapamil which significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output after 24 h of culture (Fig. 3B.3.3.2a).

Nifedipine (1, 10 and 100 μM) had no effect on PGE_2 output after 2 h of culture (Fig. 3B.3.3.2b). PGE_2 output was reduced significantly ($P < 0.05$, n=5) after 8 h and 24 h of culture by nifedipine (1 and 10 μM, but not 100 μM) (Fig. 3B.3.3.2b). Verapmail (1 μM, but not 10 and 100 μM) significantly ($P < 0.05$, n=5) reduced PGE_2 output after 2 h of culture (Fig. 3B.3.3.2b). After 8 and 24 h of culture, verapamil (10 μM, but not 1 and 100 μM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output (Fig. 3B.3.3.2b). After 24 h of culture, PGE_2 output was increased significantly ($P < 0.05$, n=5) by verapamil (100 μM, but not 1 and 10 μM) (Fig. 3B.3.3.2a).

6-Keto-$PGF_{1\alpha}$ output was unaffected by nifedipine (1, 10 and 100 μM) after 2 h of culture (Fig. 3B.3.3.2c). Nifedipine (10 and 100 μM, but not 1 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 8 h of culture, and nifedipine (1 and 10 μM, but not 100 μM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 24 h (Fig. 3B.3.3.2c). 6-Keto-$PGF_{1\alpha}$ output was unaffected by verapamil (1, 10 and 100 μM) after 2 h and 24 h of culture (Fig. 3B.3.3.2c). Verapamil (10 μM, but not 1 and 100 mM) reduced 6-keto-$PGF_{1\alpha}$ output significantly ($P < 0.05$, n=5) after 8

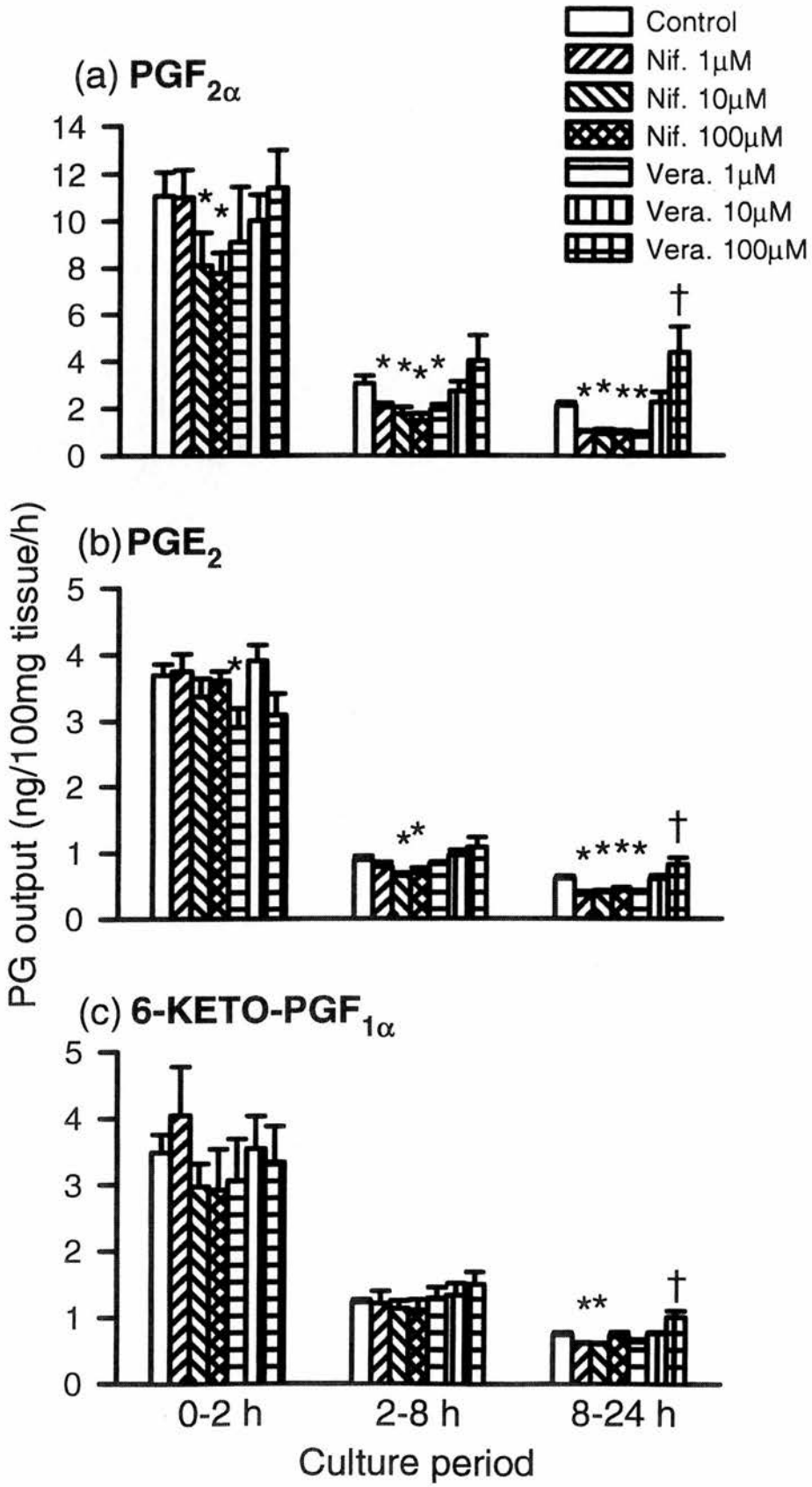


Figure 3B.3.3.1 Effects of nifedipine (1, 10 and 100 μM), verapamil (1, 10 and 100 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.

*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

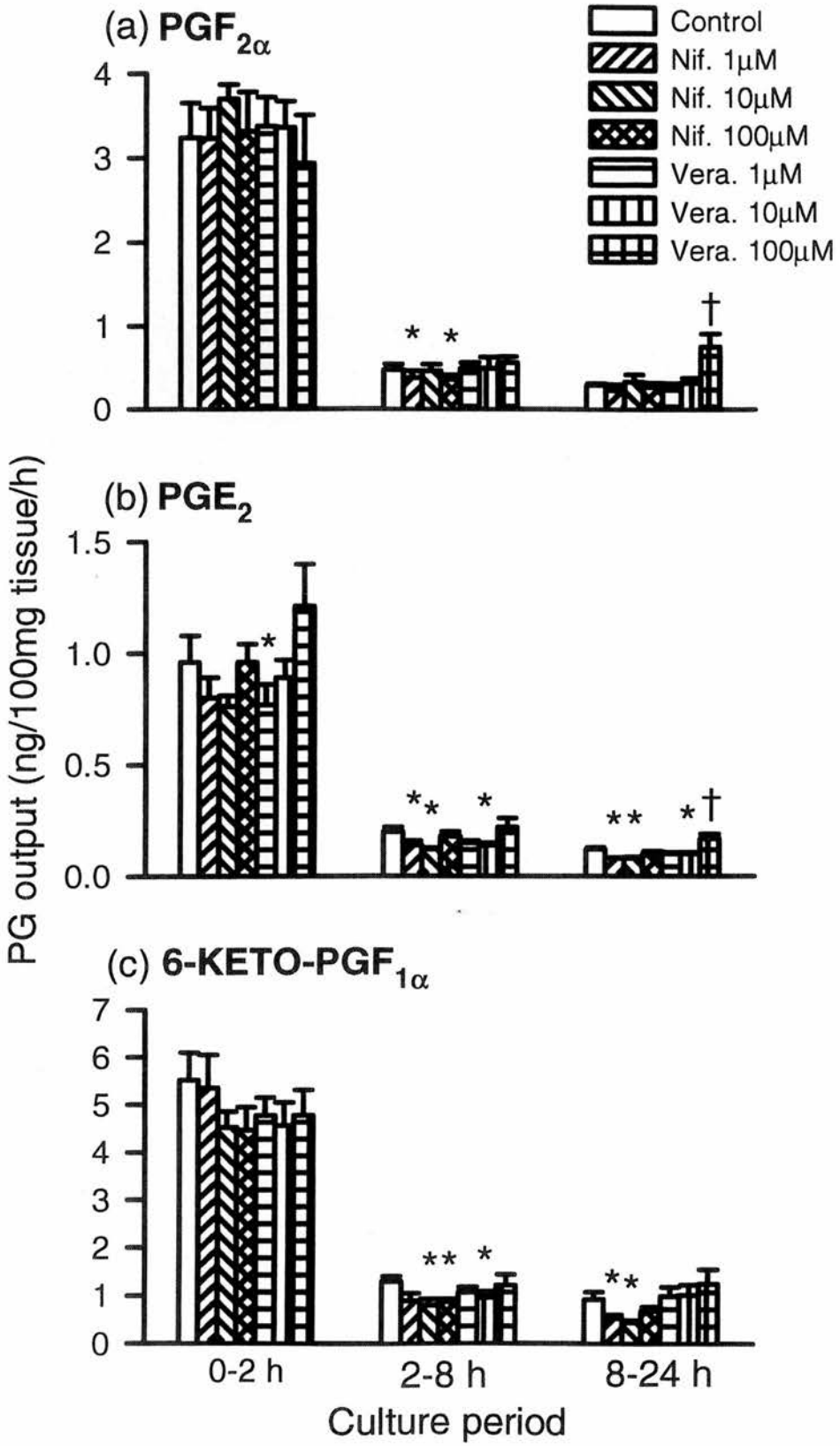


Figure 3B.3.3.2 Effects of nifedipine (1, 10 and 100 μM), verapamil (1, 10 and 100 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.

*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

h of culture (Fig. 3B.3.3.2c).

Experiment 3B.3.4 The Effect of a Stimulator of Protein Kinase C (TPA) on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

TPA (1.5, 8 and 40 nM) had no effect on $PGF_{2\alpha}$ output after 2 h of culture (Fig. 3B.3.4.1a). TPA (1.5 nM, but not 8 and 40 nM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output from the guinea-pig placenta after 8 h of culture (Fig. 3B.3.4.1a). After 24 h of culture, TPA (1.5 and 40 nM, but not 8 nM) significantly ($P < 0.05$, n=5) reduced $PGF_{2\alpha}$ output (Fig. 3B.3.4.1a).

PGE_2 output was inhibited significantly ($P < 0.05$, n=5) by TPA (1.5 nM) throughout the 24 h culture period (Fig. 3B.3.4.1b). TPA (40 nM, but not 8 nM) significantly ($P < 0.05$, n=5) inhibited PGE_2 output after 24 h of culture, but not after 2 and 8 h (Fig. 3B.3.4.1b).

TPA (1.5, 8 and 40 nM) had no significant effect on 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta after 2 h of culture (Fig. 3B.3.4.1c). After 8 and 24 h of culture, TPA (1.5 nM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output (Fig. 3B.3.4.1c). TPA (40 nM, but not 8 nM) significantly ($P < 0.05$, n=5) inhibited 6-keto-$PGF_{1\alpha}$ output after 24 h of culture, but had no significant effect after 8 h (Fig. 3B.3.4.1c).

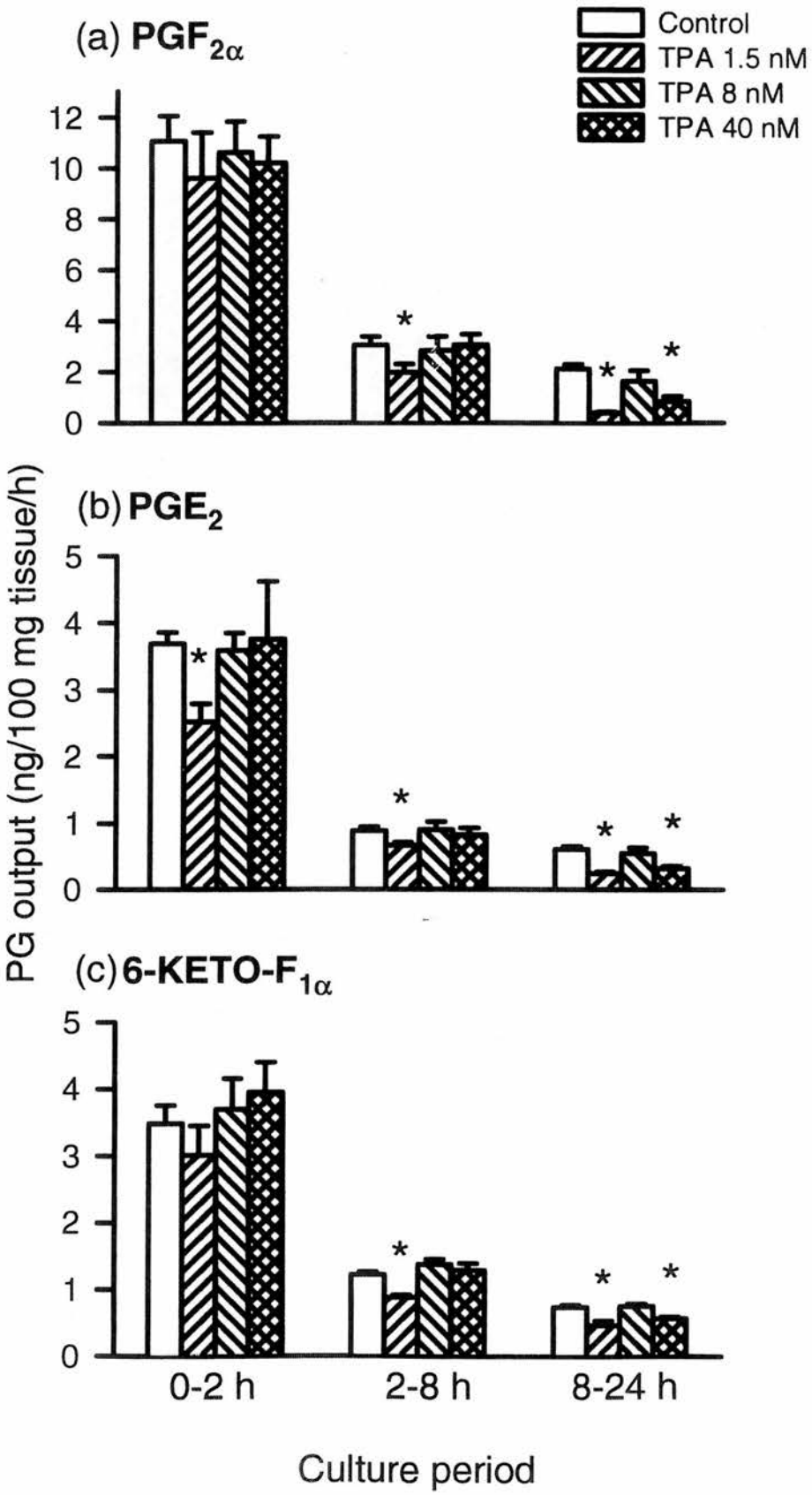


Figure 3B.3.4.1 Effect of TPA (1.5, 8 and 40 nM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$F_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.
*Significantly lower, $P < 0.05$, than corresponding control value.

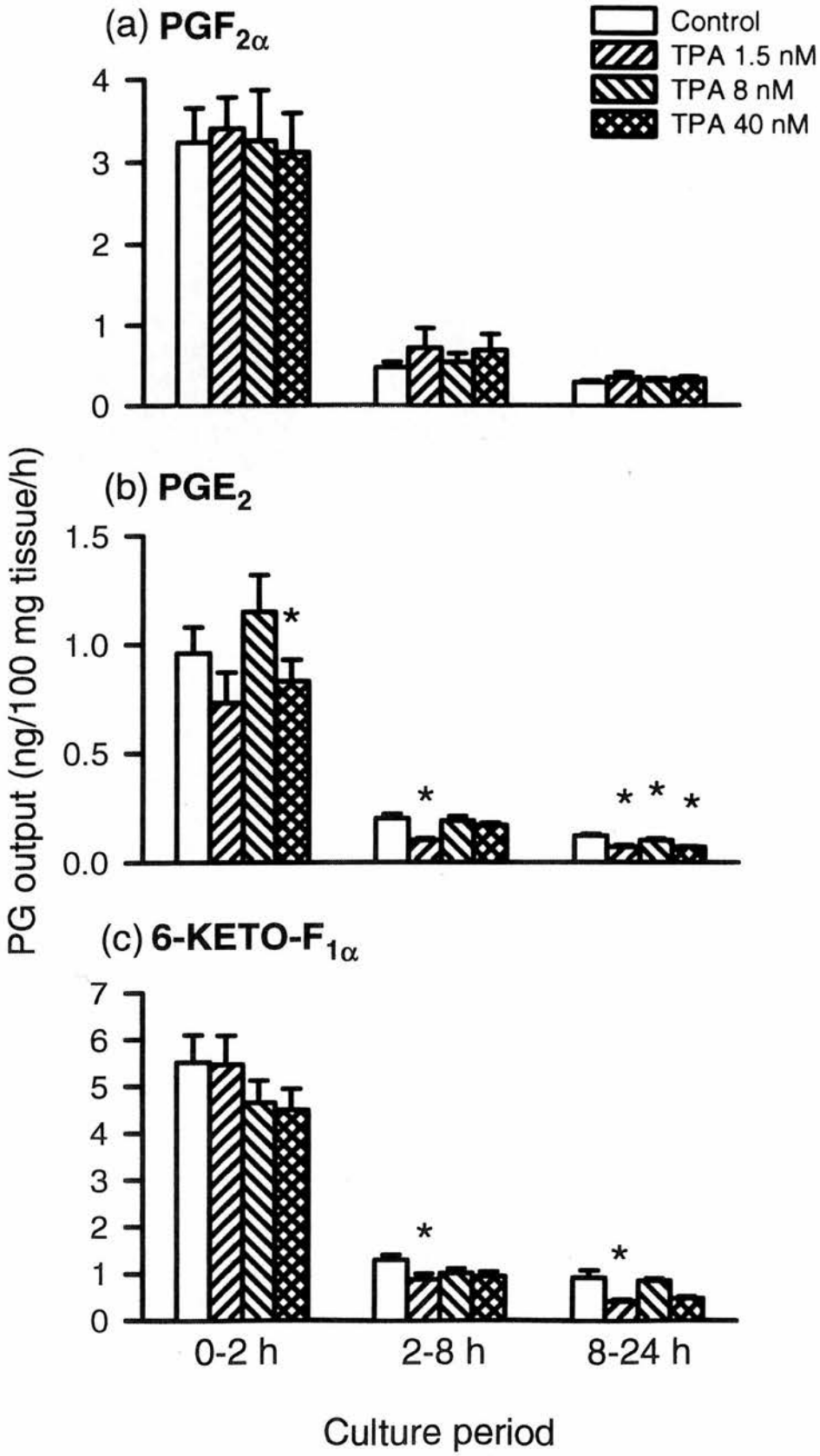


Figure 3B.3.4.2 Effect of TPA (1.5, 8 and 40 nM) on mean (±SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$F_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.
*Significantly lower, $P < 0.05$, than corresponding control value.

Sub-Placenta

TPA (1.5, 8 and 40 nM) had no effect on $PGF_{2\alpha}$ output from the guinea-pig sub-placenta throughout the 24 h culture period (Fig. 3B.3.4.2a).

PGE_2 output was significantly ($P < 0.05$, n=5) reduced after 2 h of culture by TPA (40 nM, but not 1.5 and 8 nM), and after 8 h of culture by TPA (1.5 nM, but not 8 and 40 nM) (Fig. 3B.3.4.2b). After 24 h of culture, PGE_2 output was significantly ($P < 0.05$, n=5) inhibited by TPA (1.5, 8 and 40 nM) (Fig. 3B.3.4.2b).

6-Keto-$PGF_{1\alpha}$ output was unaffected by TPA (1.5, 8 and 40 nM) after 2 h of culture (Fig. 3B.3.4.2c). 6-Keto-$PGF_{1\alpha}$ output was significantly ($P < 0.05$, n=5) reduced by TPA (1.5 nM, but not 8 and 40 nM) after 8 and 24 h of culture (Fig. 3B.3.4.2c).

Experiment 3B.3.5 The Effects of EGTA, TMB-8, Nifedipine and Verapamil on Prostaglandin Production by Day 29 Guinea-Pig Placental Homgenates.

$PGF_{2\alpha}$ production by placental homogenates was unaffected by treatment with EGTA (2 mM), TMB-8 (30 and 100 μM), nifedipine (10 and 100 μM) or verapamil (10 and 100 μM) (Fig. 3B.3.5.1a). PGE_2 production was significantly ($P < 0.05$, n=5) inhibited in the presence of EGTA (2 mM) or TMB-8 (30 μM), but treatment with TMB-8 (100 μM), nifedipine (10 and 100 μM) or verapamil (10 and 100 μM) had no significant effect (Fig. 3B.3.5.1b). 6-Keto-$PGF_{1\alpha}$ production by placental homogenates was unaffected by treatment with EGTA (2 mM), TMB-8 (30 and 100 μM), nifedipine (10 and 100 μM) or verapamil (10 and 100 μM) (Fig. 3B.3.5.1c).

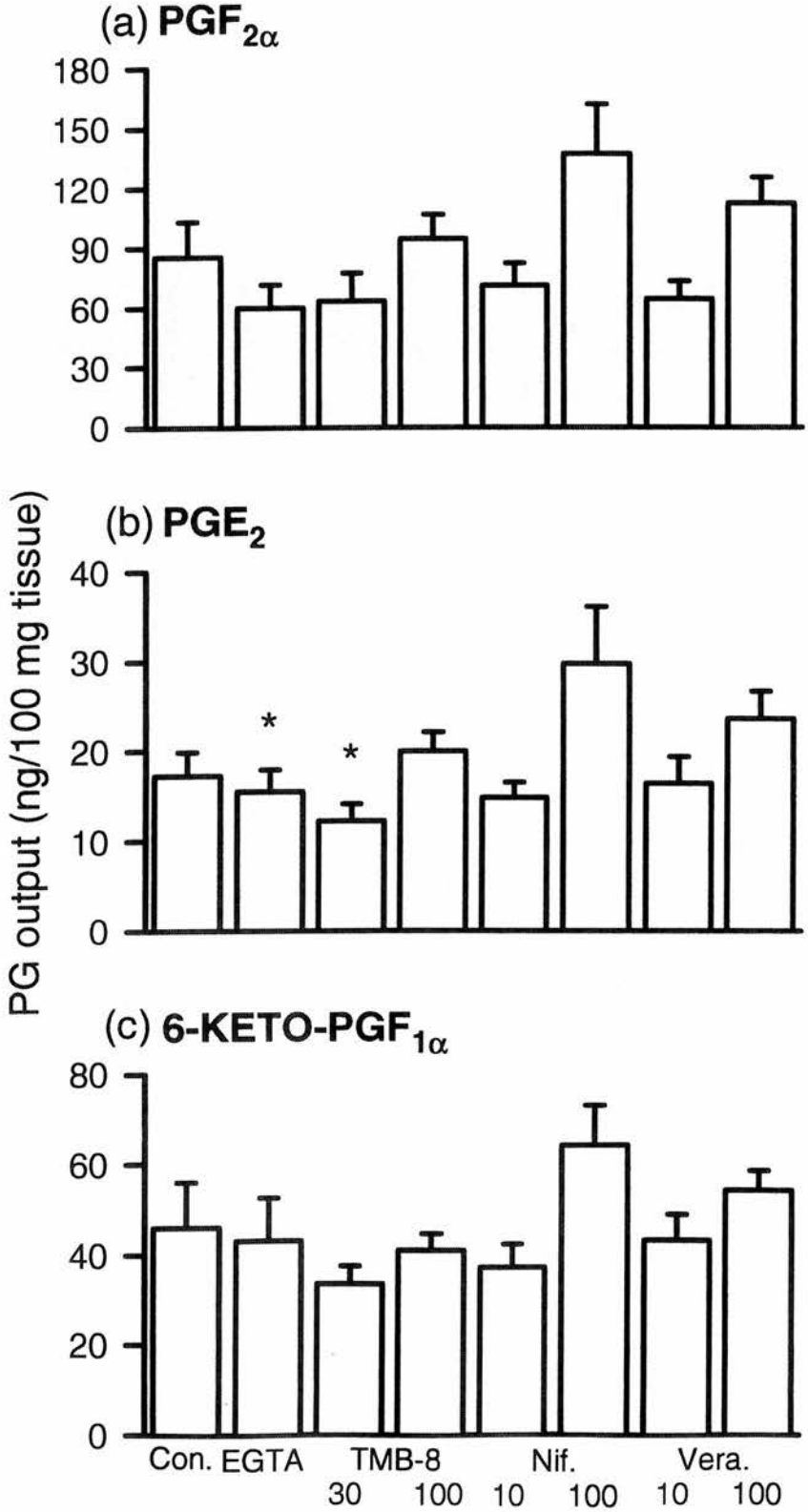


Figure 3B.3.5.1 Mean (±SEM, n=5) (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ production by guinea-pig placental homogenates in the presence of EGTA (2 mM), TMB-8 (30 and 100 μM), nifedipine (10 and 100 μM) or verapamil (10 and 100 μM).

*Significantly lower, $P < 0.05$, than corresponding control value.

Discussion:

The absence of calcium from tissue culture medium had no effect on basal outputs of $PGF_{2\alpha}$ and PGE_2 from day 29 guinea-pig placenta, cultured for 24 h. However, 6-keto-$PGF_{1\alpha}$ output was inhibited throughout the 24 h culture period, in the presence of calcium-depleted medium. This suggests that 6-keto-$PGF_{1\alpha}$ synthesis by the guinea-pig placenta is dependent on extracellular calcium, while $PGF_{2\alpha}$ and PGE_2 outputs are not. Calcium-depleted Krebs solution has previously been observed to inhibit 6-keto-$PGF_{1\alpha}$ output from superfused guinea-pig uterus, while having no affect on $PGF_{2\alpha}$ and PGE_2 outputs (Poyser, 1984). In contrast, calcium-depleted medium significantly reduced $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 29 guinea-pig sub-placenta, particularly PGE_2 output, suggesting that the synthesis of these three PGs by this tissue is dependent on extracellular calcium. Although extracellular calcium appears to be necessary for 6-keto-$PGF_{1\alpha}$ production by the day 29 guinea-pig placenta and for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the guinea-pig sub-placenta, it may only be required to replenish intracellular calcium stores.

Calcium may stimulate PLA_2 to release arachidonic acid from phospholipids in the placenta. The free arachidonic acid may then be available for PG synthesis. There are several different PLA_2 enzymes, some which rely on calcium and some which do not. The presence of a diverse family of PLA_2 enzymes provides multiple differentially regulated pathways for the important process of fatty acid turnover (Leslie, 1997).

There was very little change in $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ outputs from day 29 guinea-pig placenta and sub-placenta after 2 h of culture, in the presence of EGTA, TMB-8, TFP, W-7, nifedipine, verapamil or TPA. This suggests that the treatments used had no immediate effect on PG production and required a longer period of time to exert their effects. EGTA inhibited $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the day 29 placenta but had no effect on PGE_2 output. TMB-8 (30 μM), an intracellular calcium antagonist, inhibited PGE_2 output from day 29 placenta and also inhibited $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs, but to a lesser extent. This suggests that intracellular calcium is necessary for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the day 29 guinea-pig placenta. However, $PGF_{2\alpha}$ output from the placenta was significantly increased by TMB-8 (100 μM), which had no effect on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs. Therefore, these results suggest that TMB-8 has opposing actions on PG production by the guinea-pig placenta following 24 h of culture. Poyser (1985b) observed TMB-8 to have a similar stimulatory effect on PG output from the superfused guinea-pig uterus. The opposing actions of TMB-8 on PG production by the guinea-pig placenta, particularly at the higher concentration of 100 μM, could explain why this treatment had no inhibitory effect on PGE_2 and 6-keto-$PGF_{1\alpha}$ output from the day 29 placenta, while at a lower concentration of 30 μM, TMB-8 significantly inhibited PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs. These results indicate that PG production by the day 29 guinea-pig placenta is dependent on intracellular calcium. 6-Keto-$PGF_{1\alpha}$ output from the guinea-pig placenta also requires extracellular calcium for its synthesis. The intracellular calcium stores may be replenished by influx of calcium from the extracellular compartment.

EGTA had no significant effect on PG output from day 29 guinea-pig sub-placenta. TMB-8 (30 μM) inhibited $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the sub-placenta which suggests that intracellular calcium is required for their synthesis. At a higher concentration of 100 μM, TMB-8 also inhibited $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the sub-placenta but had no significant effect on PGE_2 output. TMB-8 had no stimulatory effect on PG output from the sub-placenta. However, the opposing actions of TMB-8 on PG production by the guinea-pig placenta may also occur in the sub-placenta and account for the lack of effect of TMB-8 on PGE_2 output. Intracellular calcium appears to be necessary for PG production by the day 29 guinea-pig sub-placenta. Therefore, PG production by the guinea-pig sub-placenta appears to be dependent on both extracellular and intracellular calcium.

TFP a calmodulin antagonist, had no effect on $PGF_{2\alpha}$ or PGE_2 output from the day 29 guinea-pig placenta, except TFP (200 μM), which significantly stimulated $PGF_{2\alpha}$ output. TFP has been observed to have opposing actions on PG output from the superfused guinea-pig uterus, and the guinea-pig endometrium maintained in culture (Poyser, 1985a; Riley & Poyser, 1987b). TFP has also been observed to stimulate arachidonic acid release from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in guinea-pig macrophages (Takenawa *et al.*, 1982). The opposing actions of TFP may account for the stimulation of PG synthesis observed from the guinea-pig placenta. W-7, another calmodulin antagonist, had no significant effect on PGE_2 output from the day 29 placenta, while $PGF_{2\alpha}$ output from the day 29 guinea-pig placenta was increased significantly in the presence of W-7. Riley & Poyser (1987b) also demonstrated that W-7 has opposing actions on PG

outputs from the cultured guinea-pig endometrium. TFP (100 μM) significantly reduced 6-keto-$PGF_{1\alpha}$ output, while at a higher concentration of 200 μM, TFP had no effect on 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta. The opposing actions of TFP could explain why 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta was significantly inhibited in the presence of TFP (100 μM), while TFP (200 μM) had no effect. 6-Keto-$PGF_{1\alpha}$ output was also significantly inhibited in the presence of W-7. These results suggest that 6-keto-$PGF_{1\alpha}$ production by the day 29 guinea-pig placenta may be dependent on calmodulin. However, $PGF_{2\alpha}$ and PGE_2 outputs were not inhibited by the presence of either of the calmodulin antagonists used, and so it is unlikely that calmodulin is necessary for their production. TFP and W-7 had opposing actions on $PGF_{2\alpha}$ output from the day 29 placenta and it remains unclear whether the treatments used had any inhibitory effect.

TFP had no inhibitory effect on $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the day 29 guinea-pig sub-placenta after 24 h of culture. However, $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs were significantly stimulated by TFP, particularly at the 200 μM concentration, and after 24 h of culture. W-7 had no effect on 6-keto-$PGF_{1\alpha}$ output but significantly increased the outputs of $PGF_{2\alpha}$ and PGE_2 from the guinea-pig sub-placenta. TFP and W-7 had no inhibitory effect on any of the PGs measured which suggests that calmodulin is not a requirement for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the day 29 sub-placenta.

Nifedipine significantly inhibited all PG outputs measured from day 29 guinea-pig placenta to some extent, particularly $PGF_{2\alpha}$ output. Verapamil (1 μM) inhibited $PGF_{2\alpha}$ and PGE_2 outputs but had no effect on 6-keto-$PGF_{1\alpha}$ output. Low

concentrations of nifedipine and verapamil are normally sufficient to block voltage-dependent calcium channels. These results suggest that the guinea-pig placenta does rely on voltage-dependent calcium channels to transport extracellular calcium to the inside of cells, even though previous experiments have shown that $PGF_{2\alpha}$ and PGE_2 production by the guinea-pig placenta is not dependent on extracellular calcium. At a concentration of 100 μM, verapamil significantly increased $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs, after 24 h of culture. Verapamil has opposing actions on PG production by the guinea-pig endometrium after 3 days of culture (Riley & Poyser, 1987b), and this also appears to be true of PG production by the guinea-pig placenta after 24 h of culture. Verapamil was observed to inhibit PG output from the placenta at a low concentration, while stimulating PG output at higher concentrations. The opposing actions of verapamil could account for the lack of inhibitory effect, especially at 10 μM. Riley & Poyser (1987b) demonstrated that nifedipine and verapamil inhibited PG output from the guinea-pig uterus and observed that these treatments may have intracellular actions on PG production by the guinea-pig uterus. This may be the case here. This could explain why extracellular calcium was not required for PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis by the placenta, while nifedipine and verapamil inhibited their outputs from the guinea-pig placenta.

Nifedipine reduced $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from day 29 sub-placenta. Verapamil had no inhibitory effect on $PGF_{2\alpha}$ ouput, while at a concentration of 10 μM, verapamil reduced PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the day 29 sub-placenta. These results suggest that extracellular calcium required for the activation of PLA_2, and hence PG synthesis does enter the guinea-pig sub-placenta

via voltage-dependent calcium channels. Verapamil has previously been observed to reduce calcium uptake by the ovine placenta by > 70% (Jones *et al.*, 1997). At a higher concentration of 100 μM, verapamil significantly increased $PGF_{2\alpha}$ and PGE_2 outputs from the sub-placenta, after 24 h of culture. This effect was also observed in the day 29 guinea-pig placenta and was attributed to the opposing effects of verapamil on PG production. The inhibitory effect of nifedipine and verapamil may also be due to an intracellular action. This has previously been observed in culture guinea-pig endometrium (Riley & Poyser, 1987b).

$PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the day 29 placenta were inhibited in the presence of TPA, particularly at the lowest concentration used. TPA had no effect on $PGF_{2\alpha}$ output from the day 29 guinea-pig sub-placenta but significantly reduced PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs. TPA is a stimulator of protein kinase C, which has previously been observed to activate PLA_2s and the mobilisation of arachidonic acid from membrane phospholipids in many tissues. However, the inhibitory action of TPA on PG output from the guinea-pig placenta and sub-placenta does not indicate activation by PKC. Therefore, it is unlikely that PKC is necessary for PG production by the guinea-pig placenta and sub-placenta. The inhibitory effect of TPA on PG output from the guinea-pig placenta and sub-placenta indicates that either PKC inhibits PG synthesis by these tissues or that TPA has an inhibitory action independent of PKC.

Since PGHS is destroyed during the synthesis of PGs (Lands, 1979), the total amount of PGs synthesised by tissue homogenates is indicative of the amount of PGHS in the tissue. The presence of EGTA, TMB-8, nifedipine or verapamil had no significant

effect on $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ production by homogenates of the guinea-pig placenta after 1 h incubation. PGE_2 production was reduced significantly in the presence of EGTA and TMB-8 (30 μM). These results suggest that PGHS activity was unaffected by the presence of EGTA, TMB-8, nifedipine and verapamil. Therefore, the inhibitory and the stimulatory effects on PG output from guinea-pig placenta and sub-placenta were not due to inhibition or stimulation of PGHS.

In summary, these results have shown that 6-keto-$PGF_{1\alpha}$ production by the day 29 guinea-pig placenta is dependent on extracellular as well as intracellular calcium and calmodulin may also be necessary for its synthesis. $PGF_{2\alpha}$ and PGE_2 production by the day 29 guinea-pig placenta appears to be dependent on intracellular calcium, but does not appear to require calmodulin. PG output from the placenta also appears to be dependent on calcium entry via voltage-dependent calcium channels. The calcium may stimulate PLA_2 to release arachidonic acid from phospholipids in the placenta. However, previous experiments have shown that extracellular calcium was not necessary for PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the placenta. Therefore, it is possible that nifedipine and verapamil may be having intracellular effects on PG production by the guinea-pig placenta. It has previously been observed that nifedipine and verapamil have similar effects on PG production by the guinea-pig endometrium (Riley & Poyser, 1987b). The activation of PKC appears not to be a requirement for $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ synthesis by the placenta.

PG production by the day 29 guinea-pig sub-placenta appears to require extracellular and intracellular calcium, probably for the activation of PLA_2 and the mobilisation of arachidonic acid from membrane phospholipids. Calcium influx may be via voltage-

dependent calcium channels. PLA_2 does not appear to be calmodulin-dependent in the guinea-pig sub-placenta as the calmodulin antagonists used had no inhibitory effect. TPA, a PKC activator, was ineffective at stimulating PG output from the guinea-pig sub-placenta, indicating that PKC is not required for $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ synthesis.

PG production by the guinea-pig placenta and sub-placenta appears to be dependent on extracellular and/or intracellular calcium. Secretory and cytosolic PLA_2 enzymes can be dependent on calcium for their activation. Mitogen-activated protein kinase (MAPK) may also be involved in PLA_2 activation, particularly cytosolic PLA_2, and this may or may not require PKC for its activation (Lin *et al.*, 1992; Qiu *et al.*, 1993; de Carvahlo *et al.*, 1996). If such a cytosolic PLA_2 is involved in PG production by guinea-pig placenta and sub-placenta, it appears to be PKC-independent. Not all PLA_2 enzymes have substrate preference for arachidonic acid but many of them have been observed to release arachidonic acid from membrane phospholipids. Several PLA_2 enzymes have been identified in the human placenta including cytosolic PLA_2 ($cPLA_2$), type II secretory PLA_2 (type II $sPLA_2$) and type IV $sPLA_2$ (Freed *et al.*, 1997; Rice *et al.*, 1994; Buhl *et al.*, 1995). Which PLA_2 enzymes are present in the guinea-pig placenta and sub-placenta and which of these are responsible for PG synthesis and which are Ca^{2+}-dependent require further investigation.

3B.4 THE ROLE OF GnRH IN PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG PLACENTA.

Introduction:

Fetal pituitary hormones, particularly gonadotrophins regulate steroid and PG synthesis by the placenta (Thorburn *et al.*, 1989). It has been speculated that gonadotrophin releasing hormone (GnRH) or luteinising hormone releasing hormone (LHRH) may have a role in the maintenance of pregnancy (Kang *et al.*, 1991). Abnormally low levels of circulating immunoreactive GnRH were observed to be associated with preterm labour and high levels of immunoreactive GnRH were observed in patients who later had post-term pregnancies (Siler-Khodr *et al.*, 1984, 1991). Previous workers have observed that GnRH stimulated $PGF_{2\alpha}$ production by human placental homogenates (Hanig *et al.*, 1982). The effects of GnRH on mid-pregnant day 29 guinea-pig placenta and sub-placenta have therefore been investigated.

Methods:

The guinea-pig placentae were removed on day 29 of pregnancy as described in Section 2.1.4. The placenta and sub-placenta were separated manually and cut into small pieces. The tissue was cultured separately on raised platforms in Petri dishes containing TCM plus gonadotrophin releasing hormone (GnRH) (see Section 2.1.2). Kilner jars contained 8 Petri dishes (i.e. 2 controls and 6 treated). The placenta and sub-placenta were treated with GnRH (100 pM, 1 nM, 10 nM, 100 nM or 1 μM). Samples were collected after 2, 8 and 24 h and stored at -20°C before being assayed

for PGs. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the paired t-test.

Results:

Experiment 3B.4.1 The Effect of GnRH on Prostaglandin Output from Day 29 Guinea-Pig Placenta and Sub-Placenta in Culture.

Placenta

$PGF_{2\alpha}$ output from the guinea-pig placenta was unaffected by GnRH (100 pM, 1, 10 and 100 nM and 1 μM) after 2 and 8 h of culture (Fig. 3B.4.1a). $PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) decreased by GnRH (100 pM and 1 nM, but not by 10 and 100 nm or 1 μM) after 24 h of culture (Fig. 3B.4.1a). GnRH (100 pM, 1, 10 and 100 nM and 1 μM) had no effect on PGE_2 output, except GnRH (10 nM) which reduced PGE_2 output signifcantly ($P < 0.05$, n=5) after 2 and 8 h of culture (Fig. 3B.4.1b). 6-Keto-$PGF_{1\alpha}$ output was reduced significantly ($P < 0.05$, n=5) after 2 h of culture by GnRH (100 pM, 100 nM and 1 μM, but not by 1 and 10 nM) (Fig. 3B.4.1c). GnRH (100 pM, 1, 10 and 100 nM and 1 μM) had no effect on 6-keto-$PGF_{1\alpha}$ output after 8 and 24 h of culture, except GnRH (10 nM) which significantly ($P < 0.05$, n=5) increased 6-keto-$PGF_{1\alpha}$ output after 24 h of culture (Fig. 3B.4.1c).

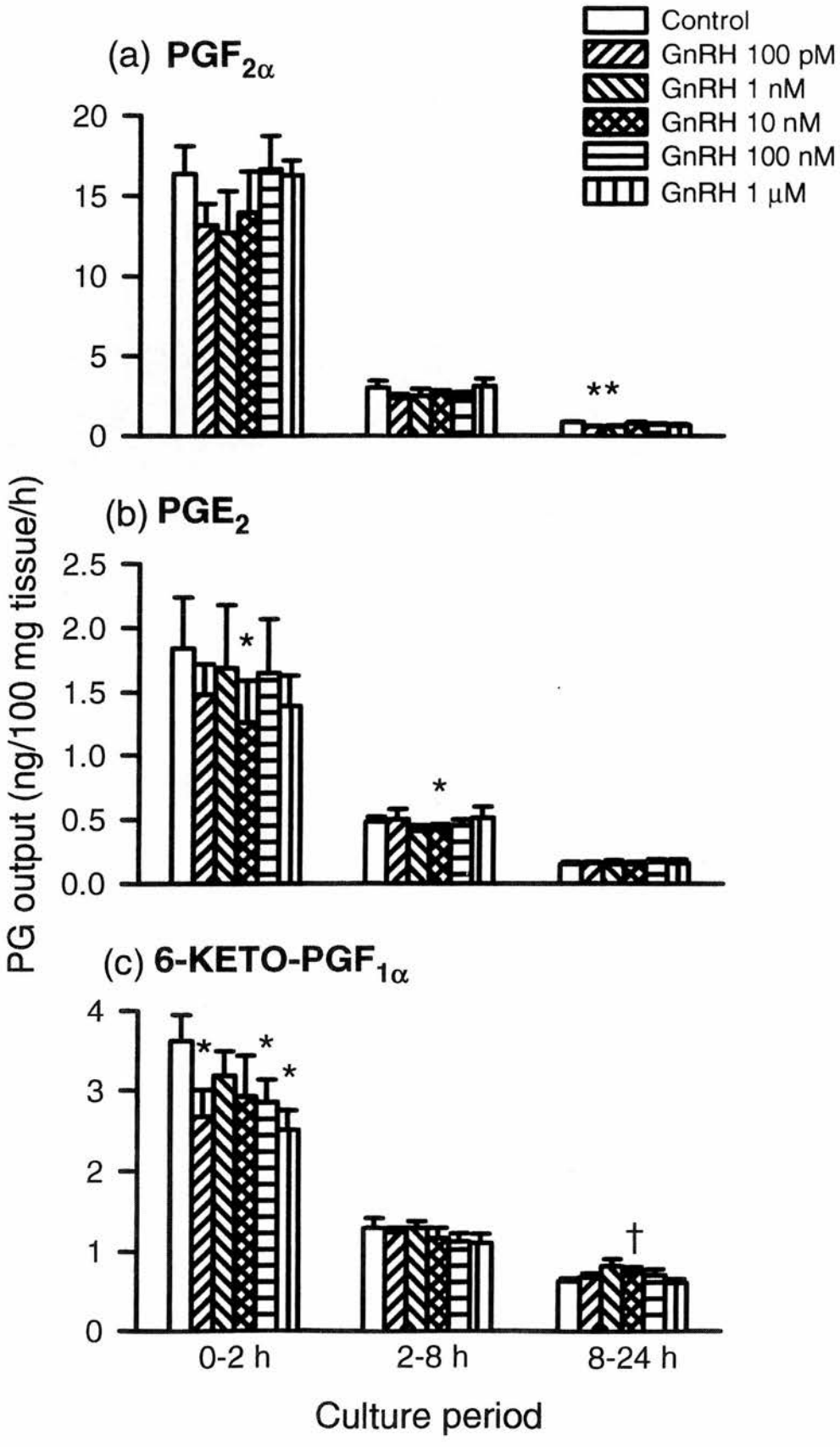


Figure 3B.4.1 Effect of GnRH (100 pM, 1 nM, 10 nM, 100 nM and 1 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig placenta cultured for 24 h.

*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

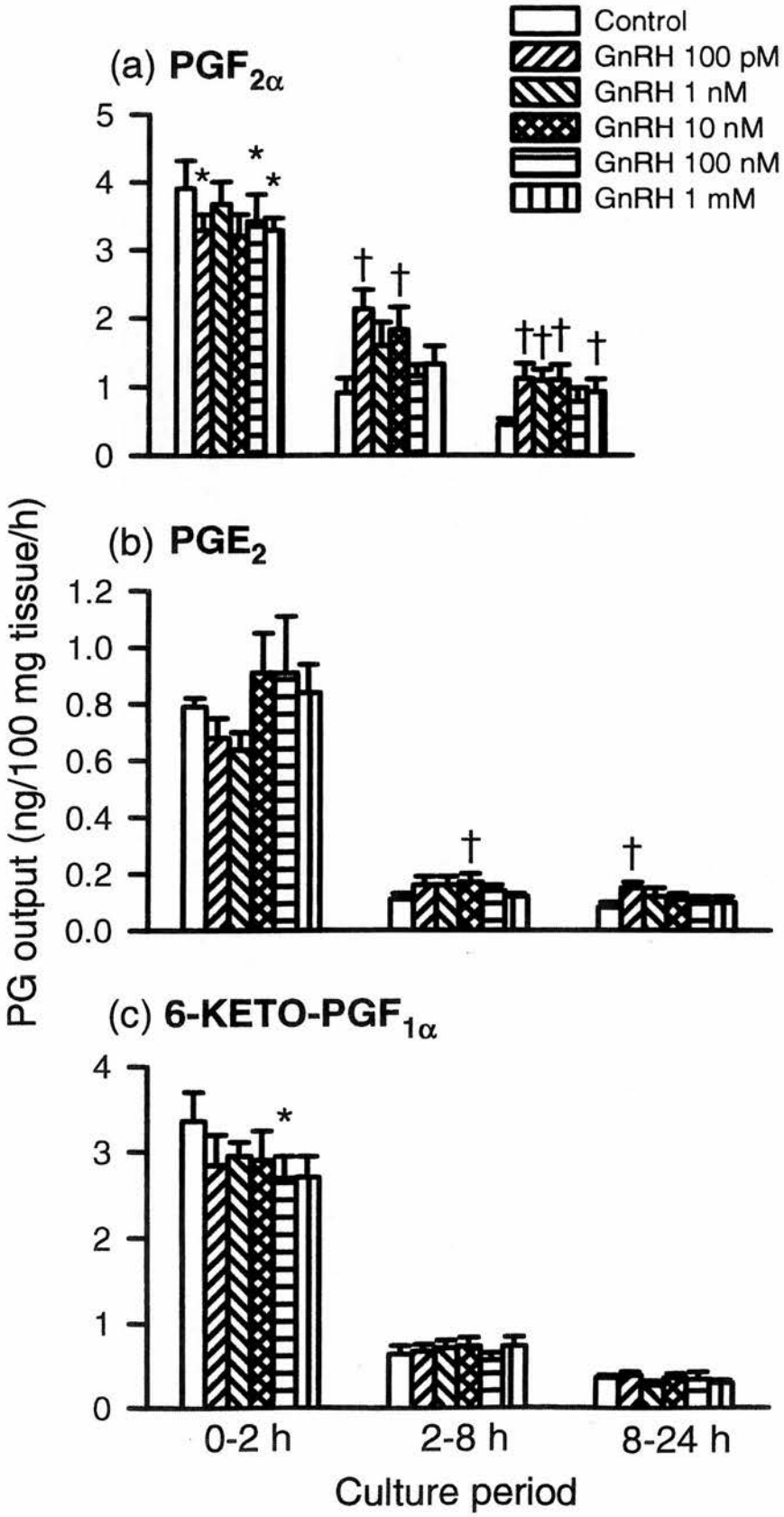


Figure 3B.4.2 Effect of GnRH (100 pM, 1 nM, 10 nM, 100 nM and 1 μM) on mean (± SEM, n=5) outputs of (a) $PGF_{2\alpha}$, (b) PGE_2 and (c) 6-KETO-$PGF_{1\alpha}$ from day 29 guinea-pig sub-placenta cultured for 24 h.
*/†Significantly lower/higher, $P < 0.05$, than corresponding control value.

Sub-Placenta

$PGF_{2\alpha}$ output was significantly ($P < 0.05$, n=5) inhibited by GnRH (100 pM, 100 nM and 1 μM, but not 1 and 10 nM) after 2 h of culture (Fig. 3B.4.2a). GnRH (100 pM and 10 nM, but not 1 and 100 nM and 1 μM) significantly ($P < 0.05$, n=5) increased $PGF_{2\alpha}$ output from the guinea-pig sub-placenta after 8 h in culture (Fig. 3B.4.2a). After 24 h of culture, GnRH (100 pM, 1 nM, 10 nM and 1 μM, but not 100 nM) increased $PGF_{2\alpha}$ output significantly ($P < 0.05$, n=5) (Fig. 3B.4.2a). PGE_2 output was unaffected in the presence of GnRH (100 pM, 1, 10 and 100 nM and 1 μM) throughout the 24 h culture period, except 10 nM GnRH which significantly ($P < 0.05$, n=5) increased PGE_2 output after 8 h of culture, and by 100 pM GnRH which increased PGE_2 output significantly ($P < 0.05$, n=5) after 24 h (Fig. 3B.4.2b). 6-Keto-$PGF_{1\alpha}$ output was unaffected by GnRH (100 pM, 1, 10 and 100 nM and 1 μM) treatment throughout the 24 h culture period, except by 100 nM GnRH which significantly ($P < 0.05$, n=5) reduced 6-keto-$PGF_{1\alpha}$ output after 2 h of culture (Fig. 3B.4.2c).

Discussion:

GnRH had no significant effect on $PGF_{2\alpha}$ output from day 29 guinea-pig placenta after 2 and 8 h of culture. However, after 24 h of culture, $PGF_{2\alpha}$ output was inhibited by the lower concentrations of GnRH used. Kang *et al.* (1991) observed that treatment of the human placenta with GnRH significantly reduced PGF and PGE outputs from the human placenta, particularly by the lower, more physiological levels of GnRH. Previous studies by the same group demonstrated that GnRH could

stimulate as well as inhibit PG output from the human placenta (Siler-Khodr *et al.*, 1986a, b). The effect of GnRH on the guinea-pig sub-placenta was more pronounced that that of the guinea-pig placenta. GnRH was observed to decrease $PGF_{2\alpha}$ output after 2 h of culture, while stimulating $PGF_{2\alpha}$ output from the sub-placenta after 8 h, and particularly after 24 h of culture. $PGF_{2\alpha}$ output from the human placenta has also been observed to increase in the presence of GnRH (Hanig *et al.*, 1982). The stimulation of $PGF_{2\alpha}$ output from the sub-placenta in the presence of GnRH may be due to an increase in PGHS synthesis as suggested by Thorburn (1991).

PGE_2 output from the placenta and the sub-placenta was largely unaffected by the presence of GnRH. GnRH had some inhibitory effect on PGE_2 output from the placenta and some stimulatory effect on PGE_2 output from the sub-placenta. Therefore, GnRH had the same effects on PGE_2 output from the placenta and the sub-placenta but to a lesser extent. GnRH significantly inhibited 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta after 2 h of culture, but had no inhibitory effects after this time. Stimulation of 6-keto-$PGF_{1\alpha}$ output from the placenta was observed after 24 h of culture in the presence of 10 nM GnRH. 6-Keto-$PGF_{1\alpha}$ output from the guinea-pig sub-placenta was mostly unaffected by the GnRH treatments used.

Bland & Donovan (1969a) postulated that placental hormones may be responsible for luteal function in the guinea-pig. It has also been suggested that these hormones may be related to human chorionic gonadotrophin. Davies *et al.* (1961) concluded that the guinea-pig sub-placenta was the source of a gonadotrophin responsible for the maintenance of pregnancy. GnRH may be stimulating the production of this gonadotrophin by the guinea-pig placenta. Humphreys *et al.* (1982) observed the

presence of gonadotrophin in the guinea-pig placenta and sub-placenta from day 10 of pregnancy, which reached a maximum after 3 weeks of gestation. This gonadotrophin could help reduce the luteolytic effects of $PGF_{2\alpha}$. Siler-Khodr *et al.* (1983, 1986a) observed an increase in PG output from the human placenta in the presence of GnRH and North *et al.* (1991) concluded that GnRH may mediate its effect via hCG. Specific binding sites (receptors) for GnRH have been demonstrated in the human placenta (Currie *et al.*, 1989) and bioactive GnRH and GnRH mRNA have also been observed (Gibbons *et al.*, 1975; Kelly *et al.*, 1991). Whether specific binding sites for GnRH are present in the guinea-pig placenta and sub-placenta which may account for the findings obtained in this study requires further investigation.

SECTION FOUR

GENERAL DISCUSSION

Prostaglandins (PGs) regulate physiological and pathological processes in all body systems including the reproductive tract. Therefore, the uterus and the placenta have been extensively studied in this thesis with respect to factors controlling PG production. In the studies presented in this thesis, Adenosine, ATP and its analogues stimulated PG production (particularly $PGF_{2\alpha}$) from day 7 superfused guinea-pig uterus and from day 7 endometrium and myometrium cultured for 24 h. This was more pronounced in the endometrium which is the main site of prostanoid synthesis in the guinea-pig uterus (Poyser, 1983). Scheimann *et al.* (1991) and Suzuki (1991) both observed increased $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig and the rabbit uterus respectively, following treatments with adenosine (A) and P2 receptor agonists. This increase in $PGF_{2\alpha}$ output could be responsible for stimulating uterine contraction in the guinea-pig uterus as suggested by Smith *et al.* (1988) and Piper & Hollingsworth (1996). Piper & Hollingsworth (1996) proposed that PGs produced by the endometrium are involved in ATP-induced contractions of the non-pregnant guinea-pig uterus after demonstrating that indomethacin, a non-selective PGHS inhibitor, and removal of the endometrium inhibited ATP-induced contraction of the guinea-pig uterus. Suramin, a non-selective P2 receptor antagonist, significantly inhibited the ATP-induced increase in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus (see Section 3A.3). This effect of suramin on ATP-

stimulated $PGF_{2\alpha}$ production suggests that ATP exerts its effect via P2 receptors. A and P2 receptors have previously been observed in the guinea-pig uterus (Smith *et. al.*, 1988). However, as well as acting on P2 receptors, ATP may indirectly be stimulating adenosine receptors after being broken down to adenosine by ecto-nucleotidases, especially after 24 h of culture (see Section 3A.4). However, the non-selective A receptor antagonist, 8-sulphophenyltheophylline, had no effect on ATP-induced $PGF_{2\alpha}$ output from guinea-pig uterus (see Section 3A.3 and 3A.4), suggesting that ATP was probably exerting its action predominantly by activating P2 receptors.

Previous workers have disagreed over the involvement of PGs in spasm of the guinea-pig uterus mediated by A receptors (Scheimann *et* al., 1991; Bradley *et al.*, 1993). Bradley *et al.* (1993) proposed that cyclooxygenase products were not involved in adenosine-induced contractions of the non-pregnant guinea-pig uterus, while Scheimann *et al.* (1991) concluded that activation of A receptors in the non-pregnant guinea-pig uterus leads to stimulation of a cyclooxygenase product, resulting in contraction of uterine smooth muscle. The present study indicates that PGs may be involved in adenosine-induced spasm of the guinea-pig uterus. Adenosine was observed to stimulate $PGF_{2\alpha}$ output from the guinea-pig uterus superfused *in vitro* (see Section 3A.1), and this stimulation by adenosine was inhibited by the A receptor antagonist, 8-sulphophenyltheophylline. Therefore, it is clear that ATP- and adenosine-induced increases in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus are receptor mediated responses as the actions of ATP and adenosine were significantly reduced by the P2 and A receptor antagonists,

suramin and 8-sulphophenyltheophylline, respectively (see Section 3A.3). P2Y purinoceptors have previously been observed in the guinea-pig uterus and, more specifically, in the endometrium (Piper & Hollingsworth, 1996). P2Y receptors are G protein-linked receptors (Webb *et al.*, 1993). They have been observed to couple via G proteins to phospholipase A_2 (PLA_2) (see Axelrod *et al.*, 1988) and phospholipase C (PLC) in a number of tissues (see Piper & Hollingsworth, 1996). PLA_2 is well documented as the enzyme responsible for arachidonic acid release from membrane phospholipids, and it is possibly responsible for increased PG synthesis from the superfused guinea-pig uterus stimulated by adenosine, ATP and ATP analogues. However, activation of PLC can also lead to the mobilisation of free arachidonic acid from inositol phospholipids (IP). Diacylglycerol (DAG) is one of the breakdown products of IP hydrolysis by PLC. Arachidonic acid can be released from DAG by the sequential action of diacylglycerol lipase and monoacylglycerol lipase. Previous workers have suggested that adenosine- and ATP-induced stimulation of PG synthesis could be linked to IP hydrolysis in the guinea-pig and the rabbit uterus (Scheimann *et al.*, 1991; Suzuki, 1991). ATP has been observed to stimulate DAG formation in the rabbit uterus, indicative of PLC activation (Suzuki, 1991), and stimulation of adenosine receptors leads to rapid accumulation of inositol phosphates in the guinea-pig uterus (Scheimann *et al.*, 1991). Thus, it is also possible that adenosine, ATP and ATP analogues stimulate $PGF_{2\alpha}$ output from the guinea-pig uterus by the actions of PLC, diaglyceride lipase and monoglyceride lipase.

Diacylglycerol (DAG) can also activate protein kinase C (PKC), a second messenger system that has been implicated in stimulation of PGHS expression (DeWitt, 1991). PKC stimulates a kinase cascade leading to mitogen-activated protein kinase

(MAPK) activation (Qiu & Leslie, 1994). MAPK activates transcription factors (Karin & Hunter, 1995), and in particular NF-κB which leads to the induction of expression of many immediate early genes (Baeuerle, 1991). NF-κB has been observed to increase PGHS-2 synthesis in lipopolysaccharide (LPS)-stimulated macrophages (Hwang *et al.*, 1997). Previous studies have shown the presence of PGHS-2 in the guinea-pig endometrium by Western blotting (Naderali & Poyser, 1994). It was demonstrated that PGHS-2 is the main functional form of PGHS in the guinea-pig endometrium and myometrium (Naderali & Poyser, 1996a). Thus, the increase in PG synthesis by the guinea-pig endometrium observed after 24 h of culture may be due to adenosine, ATP and ATP analogues increasing the synthesis of PGHS-2 by processes involving the activation of PKC, MAPK and NF-κB.

MAPK also activates $cPLA_2$ (Lin *et al.*, 1993) which, in turn, results in increased PG synthesis. Inositol 1,4,5-triphosphate (IP_3) is a specific breakdown product of PLC-mediated phosphatidyl-4,5-biphosphate (PIP_2) hydrolysis and is a unique second messenger that releases calcium from the endoplasmic reticulum of several cell types, including the sarcoplasmic reticulum of smooth muscle cells (Schrey *et al.*, 1988). Scheimann *et al.* (1991) suggest that IP_3 is capable of releasing calcium from the guinea-pig uterine intracellular stores. Increased calcium concentration can activate PLA_2 enzymes, which stimulate arachidonic acid mobilisation and increased PG synthesis. Therefore, adenosine, ATP and ATP analogues may initiate arachidonic acid release via IP_3-induced IP_3 calcium release, with the calcium activating $cPLA_2$. Overall, there are a number of possible mechanisms for arachidonic acid release and stimulation of PG synthesis in the guinea-pig uterus by ATP, ATP analogues and adenosine.

In order to further elucidate the mechanisms of actions of ATP, ATP analogues and adenosine on PG production by the non-pregnant guinea-pig uterus, it is necessary to examine the effect of the A and P2 antagonists, 8-sulphophenyltheophylline and suramin, on PG production by cultured endometrium and myometrium. This will determine whether these compounds have any significant effect on PG outputs from the guinea-pig uterus on their own. The use of a PLA_2 antagonist would be useful to determine whether ATP and adenosine-induced increases in $PGF_{2\alpha}$ output from the superfused guinea-pig uterus are caused by activation of PLA_2. Further studies are required to elucidate which pathways are actually involved in PG synthesis by the guinea-pig uterus in response to adenosine and ATP.

It is well documented that $PGF_{2\alpha}$ is the luteolytic hormone in many species and that $PGF_{2\alpha}$ output from the guinea-pig uterus increases from day 11 of the oestrous cycle (see Poyser, 1995). Measurement of the $PGF_{2\alpha}$ metabolite, 5α, 7α-dihydroxy-11-ketotetranor-prostanoic acid, in guinea-pig urine showed that $PGF_{2\alpha}$ production by the guinea-pig uterus is suppressed during pregnancy during the time luteolysis takes place if the animal is not pregnant (Granström & Kindahl, 1976). However the concentration of $PGF_{2\alpha}$ metabolite in the guinea-pig urine rapidly increases at approximately day 25 of gestation, and remains high as pregnancy proceeds. Removal of the pregnant uterus during pregnancy results in a rapid fall in the urinary concentration of $PGF_{2\alpha}$ metabolite (Granström & Kindahl, 1976). Therefore, the pregnant guinea-pig uterus or its contents must be the source of the $PGF_{2\alpha}$ produced during pregnancy. $PGF_{2\alpha}$ output from the guinea-pig placenta increased 8-fold

between days 22 and 29 of pregnancy after 2 h of culture, and remained higher on day 29 when compared to day 22 throughout the 24 h culture period (see Section 3B.1.1). $PGF_{2\alpha}$ output from the guinea-pig sub-placenta was also high after 2 h of culture and was 2-fold higher on day 29 than on day 22 (see Section 3B.1.1). Previous experiments by Norman & Poyser (1998b) showed that $PGF_{2\alpha}$ output from the guinea-pig placenta increases 15-fold between days 22 and 36 of pregnancy. Norman & Poyser (1998b) speculated that this increase in $PGF_{2\alpha}$ output may account for the increase in $PGF_{2\alpha}$ metabolite observed in guinea-pig urine from day 25 of pregnancy. It has previously been observed that the placenta is a major site of PG biosynthesis during pregnancy (Keirse, 1979). This study has shown that PGFM output from the guinea-pig placenta was very high. This high output of PGFM could mean that the basal $PGF_{2\alpha}$ outputs from day 22 and day 29 guinea-pig placentae have been underestimated and that perhaps the metabolite levels should have been measured throughout. When the outputs of $PGF_{2\alpha}$ from day 22 and day 29 are considered together with the PGFM outputs from day 22 and day 29 placenta, it does appear that there may be a decrease in metabolism within the first 2 h of culture. After 2 h of culture, metabolism does appear to increase with increased $PGF_{2\alpha}$ production by the day 29 guinea-pig placenta. The metabolism of $PGF_{2\alpha}$ may be an important mechanism for maintaining pregnancy and may act as a barrier to prevent primary active PGs getting to the myometrium and causing unwanted contraction of the mid-pregnant uterus. It has been suggested that the guinea-pig placenta has a decreased capacity for PG metabolism in late gestation (Moussard *et al.*, 1986). However, day 29 is considered as mid-pregnancy. The high metabolite levels

observed in guinea-pig urine by Granström & Kindahl (1976) suggest that metabolite levels may increase with $PGF_{2\alpha}$ production by the guinea-pig placenta. However, further investigation is required to determine whether metabolism does actually decrease as pregnancy proceeds.

$PGF_{2\alpha}$ output from both the placenta and sub-placenta was significantly greater than the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from the guinea-pig placenta and sub-placenta. The PG synthesising capacity of guinea-pig placental homogenates increased between days 22 and 29 of pregnancy, which suggests that the increase in placental PG output between these two days is due to increased PGHS synthesis. Schellenberg & Kirby (1997) propose that PGHS is the rate-limiting step for PG production by the guinea-pig placenta, and previous studies have provided evidence which suggests that PGHS activity in the placenta gradually increases during pregnancy (Moussard *et al.*, 1986; Rice *et al.*, 1988, 1989). Up-regulation of PGHS expression is a common effect of many agents that stimulate PG synthesis, including growth factors, hormones, interleukins and phorbol esters (see DeWitt, 1991).

It has been suggested that PGHS activity plays a key role in the maintenance of pregnancy in humans (Kelly, 1994). Two PGHS enzymes have been identified, cloned and sequenced (DeWitt & Smith, 1988; Merlie *et al.*, 1988; Kujubu *et al.*, 1991; O'Banion *et al.*, 1992). Previous workers have observed PGHS-2 to be the predominant enzyme involved in PG synthesis by the ovine and the human placenta (Wimsatt *et al.*, 1993; Freed *et al.*, 1995; Rice *et al.*, 1995; Macchia *et al.*, 1997). Non-steroidal anti-inflammatory drugs (NSAIDS) inhibit the cyclooxygenase activity of PGHS enzymes. The effects of indomethacin (a non-selective inhibitor of PGHS) and NS-398 (a selective inhibitor of PGHS-2) were studied to determine which

PGHS enzyme was responsible for the increase in PG output from the guinea-pig placenta and sub-placenta between days 22 and 29 of pregnancy. Indomethacin and NS-398 were equipotent at inhibiting $PGF_{2\alpha}$ output from the day 22 and day 29 guinea-pig placenta and sub-placenta. This functional study showed that PGHS-2 is the predominant enzyme responsible for $PGF_{2\alpha}$ synthesis by day 22 and 29 guinea-pig placenta and sub-placenta. PGHS-2 is not normally present in cells. So PGHS-2 expression may be induced in the placenta as pregnancy proceeds. It appears that there is a large increase in PGHS-2 expression in guinea-pig placenta between days 22 and 29 of pregnancy. Indomethacin was more effective in inhibiting PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig placenta and sub-placenta than NS-398. This suggests that both isoforms of the PGHS enzyme (PGHS-1 and PGHS-2) are involved in PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis by day 22 and day 29 guinea-pig placenta and sub-placenta. PGHS-1 and PGHS-2 are often co-expressed in the same cell, and it has been proposed that PGHS-2 may co-localise with PGHS-1 to augment the function of PGHS-1 or substitute for it if PGHS-1 is lacking (Smith *et al.*, 1996). PGHS-2 is also associated with cell differentiation and replication (Otto & Smith, 1995), which could be an advantage for the developing fetus and placenta. The signal transduction mechanism responsible for stimulation of PGHS expression and increased PG synthesis by the guinea-pig placenta and sub-placenta as pregnancy proceeds requires further investigation. The availability of specific PGHS-1 inhibitors would help to determine the degree of importance of either PGHS-1 or PGHS-2 with respect to prostaglandin production by the guinea-pig placenta and sub-placenta as pregnancy proceeds.

PGHS is destroyed during PG biosynthesis, therefore fresh enzyme must be provided if biosynthesis is to be maintained (Lands, 1979). The process of protein synthesis is necessary for the continued production of PGHS, the enzyme responsible for the conversion of arachidonic acid to PGs. Protein synthesis inhibitors (cycloheximide, puromycin and actinomycin D) had an inhibitory effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 29 guinea-pig placenta and sub-placenta after 24 h of culture (see Section 3B.2.1). Therefore, PG production by day 29 guinea-pig placenta and sub-placenta may be dependent on the fresh synthesis of proteins. Protein synthesis inhibitors have previously been observed to inhibit the output of PGs, particularly $PGF_{2\alpha}$, from guinea-pig endometrium on days 7 and 15 of the oestrous cycle (Riley & Poyser, 1989). In addition the protein synthesis inhibitors prevented the synthesis of PGHS in the guinea-pig endometrium during culture (Riley & Poyser, 1989). These results suggest that protein synthesis is necessary for the production of PGHS enzymes and that protein synthesis may be a requirement for the production of PGs by day 29 guinea-pig placenta and sub-placenta. The protein synthesis inhibitors used, particularly cycloheximide, were also observed to stimulate PG output from the guinea-pig placenta and sub-placenta, especially $PGF_{2\alpha}$ output (see Section 3B.2.1). This stimulatory effect was most pronounced when higher concentrations of the protein synthesis inhibitors were used. Riley & Poyser (1989) have previously observed that protein synthesis inhibitors stimulate PG production by the superfused guinea-pig uterus, particularly cycloheximide. Naderali & Poyser (1996b) have also observed that cycloheximide was not as effective in inhibiting PG output from cultured guinea-pig endometrium as actinomycin D and puromycin

because it has a short-term stimulatory effect. It has also been shown that expression of PGHS mRNA can be superinduced by cycloheximide (DeWitt *et al.*, 1990; Maier *et al.*, 1990). This stimulation of PGHS may account for the observed increase in $PGF_{2\alpha}$ output from the placenta and sub-placenta after 24 h of culture in the presence of cycloheximide. The stimulatory effects of cycloheximide, puromycin and actinomycin D may account for the lack of inhibition observed, particularly regarding PGE_2 output from the guinea-pig placenta. Overall, this study has shown that protein synthesis is necessary for the continued production of PGs.

Phospholipase (PL) A_2 enzymes are usually responsible for arachidonic acid release from membrane phospholipids, and some of these PLA_2 enzymes are dependent on calcium for their activation. It is well documented that arachidonic acid release for PG synthesis is triggered in cells by many calcium mobilising agonists (Clark *et al.*, 1995). Therefore the calcium requirement for PG production by day 29 guinea-pig placenta and sub-placenta was investigated. Culturing day 29 guinea-pig placenta for 24 h in the presence of calcium-depleted culture medium had no significant effect on $PGF_{2\alpha}$ and PGE_2 outputs. Therefore, $PGF_{2\alpha}$ and PGE_2 production by day 29 guinea-pig placenta does not appear to be dependent on extracellular calcium. 6-Keto-$PGF_{1\alpha}$ output from the guinea-pig placenta was significantly reduced throughout culture in the presence of calcium-depleted medium. This suggests that 6-keto-$PGF_{1\alpha}$ production by the day 29 guinea-pig placenta is dependent on extracellular calcium. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig sub-placenta were significantly reduced in the presence of calcium-depleted culture medium,

suggesting that extracellular calcium is a requirement for their synthesis by the sub-placenta. However, the extracellular calcium required for 6-keto-$PGF_{1\alpha}$ production by the placenta and $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the sub-placenta may only be needed to replenish intracellular calcium stores.

To determine whether intracellular calcium was required for PG production by day 29 guinea-pig placenta and sub-placenta, the effects of TMB-8, an intracellular calcium antagonist, was investigated. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the guinea-pig placenta and sub-placenta appear to be dependent on intracellular calcium since TMB-8 inhibited $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the placenta and the sub-placenta, particularly after 24 h of culture. TMB-8 was observed to have opposing actions on $PGF_{2\alpha}$ production by the placenta, inhibiting $PGF_{2\alpha}$ output at a low concentration and stimulating $PGF_{2\alpha}$ output at a higher concentration (see Section 3B.3.2). The opposing actions of TMB-8 shown here have been seen previously by Poyser (1985b), who observed that TMB-8 stimulated PG output from the superfused guinea-pig uterus. Nevertheless, the findings in the present study support that PG production by day 29 guinea-pig placenta and sub-placenta requires extracellular and/or intracellular calcium, probably for the activation of PLA_2 and the consequent mobilisation of arachidonic acid from membrane phospholipids. This free arachidonic acid may then be available for PG synthesis. There are several different types of PLA_2, some of which are dependent on calcium for their activation and some of which are not. The presence of this diverse family of PLA_2 enzymes provides multiple differentially regulated pathways for the important process of fatty acid turnover (Leslie, 1997). To date only four types of

PLA_2 enzyme have been identified in the human placenta; $cPLA_2$, type II $sPLA_2$, type IV $sPLA_2$ and type V $sPLA_2$ (Seilhamer *et al.*, 1989; Bennett *et al.*, 1993; Chen *et al.*, 1994; Buhl *et al.*, 1995; Freed *et al.*, 1997; Rice *et al.*, 1998). Type II $sPLA_2$ appears to be the dominant type in the human placenta (Freed *et al.*, 1997). Which PLA_2 enzymes are present in the guinea-pig placenta and sub-placenta requires further investigation.

Calmodulin is a calcium-binding protein and functions in a variety of cells (Cheung, 1980). In some tissues PLA_2 can be a calmodulin-dependent enzyme (Wong & Cheung, 1979; Maskowitz *et al.*, 1983). Calmodulin antagonists, TFP and W-7, had no significant inhibitory effect on $PGF_{2\alpha}$ and PGE_2 outputs from the guinea-pig placenta or the sub-placenta (see Section 3B.3.2). Therefore, it is unlikely that $PGF_{2\alpha}$ and PGE_2 production by the guinea-pig placenta requires calmodulin. TFP and W-7 significantly reduced 6-keto-$PGF_{1\alpha}$ output from the placenta in culture. They have no effect on 6-keto-$PGF_{1\alpha}$ output from the sub-placenta. Therefore, 6-keto-$PGF_{1\alpha}$ production by the guinea-pig placenta, but not the sub-placenta, appears to require calmodulin for its synthesis. The calmodulin antagonists used (TFP and W-7) had stimulatory effects, as well as some inhibitory effects, on PG production by the guinea-pig placenta and sub-placenta. TFP and W-7 had similar stimulatory effects on PG output from the superfused guinea-pig uterus and cultured guinea-pig endometrium (Poyser, 1985a; Riley & Poyser, 1987b). The mechanisms involved in this stimulatory action of TFP and W-7 remain unclear.

This thesis has shown that calcium is a requirement for PG production by the guinea-pig placenta and sub-placenta, whether it is of extracellular and/or intracellular

origin. Calcium influx may be via voltage-dependent calcium channels. $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the placenta and the sub-placenta appear to be dependent on calcium entry via voltage-dependent calcium channels as the voltage-dependent calcium channel blockers, nifedipine and verapamil, reduced $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the placenta and sub-placenta after 24 h of culture. Nifedipine had a marked effect. These results suggest that calcium of extracellular origin may enter cells of the guinea-pig placenta and sub-placenta via voltage-dependent calcium channels. Verapamil has previously been observed to reduce calcium uptake by the ovine placenta by > 70% (Jones *et al.*, 1997). Once inside the cell, the calcium may stimulate PLA_2 to release arachidonic acid from membrane phospholipids in the placenta or replenish intracellular calcium stores. However, nifedipine and verapamil may be acting intracellularly, as removal of extracellular calcium from tissue culture medium (TCM) had no effect on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig placenta. Riley & Poyser (1987b) suggest that calcium channel blockers may be inhibiting PG output from the guinea-pig uterus by intracellular actions. This could explain why nifedipine and verapamil had an inhibitory effect on PG output from the guinea-pig placenta and sub-placenta. Verapamil, at high concentrations, had a stimulatory effect on PG output from the guinea-pig placenta and sub-placenta after 24 h of culture. Riley & Poyser (1987b) have previously observed similar opposing actions of verapamil on PG output from the guinea-pig endometrium.

It is well documented that phorbol esters stimulate the activation of protein kinase C (PKC). It has been reported that phorbol esters also stimulate PGHS-2 mRNA

expression in cultured human amnion cells (Zakar *et al.*, 1996). Mitogen-activated protein kinase (MAPK) may also be involved in PLA_2 activation, particularly cytosolic PLA_2, and this may or may not require PKC for its activation (Lin *et al.*, 1992; Qiu *et al.*, 1993; de Carvahlo *et al.*, 1996). The activation of PKC does not appear to be a requirement for $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ synthesis by the guinea-pig placenta or sub-placenta. The PKC activator, TPA, had no stimulatory effect on PG output from the guinea-pig placenta or sub-placenta after 24 h of culture (see Section 3B.3.4). If cytosolic PLA_2 is involved in PG production by guinea-pig placenta and sub-placenta, it appears to be PKC-independent. However, this does not exclude a role for MAPK, which does not always require PKC for its activation.

It has been proposed (Kang *et al.*, 1991) that gonadotrophin releasing hormone (GnRH) may have a key role to play in the maintenance of pregnancy. Kang *et al.* (1991) observed that treatment of the human placenta with GnRH significantly reduced PGF and PGE outputs from the human placenta, particularly by the lower, more physiological levels of GnRH. Previous studies by the same group demonstrated that GnRH could stimulate as well as inhibit PG output from the human placenta (Siler-Khodr *et al.*, 1986a, b). In another study $PGF_{2\alpha}$ output from the human placenta was observed to increase in the presence of GnRH (Hanig *et al.*, 1982). GnRH had no significant effect on $PGF_{2\alpha}$ output from day 29 guinea-pig placenta after 2 and 8 h of culture. However, after 24 h of culture, $PGF_{2\alpha}$ output was inhibited by the lower concentrations of GnRH used. The effect of GnRH on $PGF_{2\alpha}$ output from the guinea-pig sub-placenta was more pronounced than its effect on the guinea-pig placenta. GnRH was observed to decrease $PGF_{2\alpha}$ output after 2 h of

culture, while stimulating $PGF_{2\alpha}$ output from the sub-placenta after 8 h and, particularly, after 24 h of culture. The stimulation of $PGF_{2\alpha}$ output from the sub-placenta in the presence of GnRH may be due to an increase in PGHS synthesis (Thorburn, 1991).

PGE_2 output from the placenta and the sub-placenta was largely unaffected by the presence of GnRH. GnRH had some inhibitory effect on PGE_2 output from the placenta and some stimulatory effect on PGE_2 output from the sub-placenta. Therefore, GnRH had the same effects on PGE_2 output as on $PGF_{2\alpha}$ output from the placenta and the sub-placenta but to a lesser extent. GnRH significantly inhibited 6-keto-$PGF_{1\alpha}$ output from the guinea-pig placenta after 2 h of culture, but had no inhibitory effects after this time. Stimulation of 6-keto-$PGF_{1\alpha}$ output from the placenta was observed after 24 h of culture in the presence of 10 nM GnRH. 6-Keto-$PGF_{1\alpha}$ output from the guinea-pig sub-placenta was mostly unaffected by the GnRH treatments used.

Bland & Donovan (1969) postulated that placental hormones may be responsible for luteal function. It has also been suggested that in the guinea-pig these placental hormones may be related to human chorionic gonadotrophin (Humphreys *et al.*, 1982). Davies *et al.* (1961) concluded that the guinea-pig sub-placenta was the source of a gonadotrophin responsible for the maintainence of pregnancy. GnRH, produced by the placenta, may be stimulating this gonadotrophin in the guinea-pig. Humphreys *et al.* (1982) observed the presence of gonadotrophin in the guinea-pig placenta and sub-placenta from day 10 of pregnancy, which reached a maximum after 3 weeks of gestation. This gonadotrophin could help reduce the luteolytic

effects of $PGF_{2\alpha}$. Siler-Khodr *et al.* (1983, 1986a) observed an increase in PG output from the human placenta in the presence of GnRH and North *et al.* (1991) concluded that GnRH may mediate its effect via hCG. Specific binding sites (receptors) for GnRH have been demonstrated in the human placenta (Currie *et al.*, 1989) and bioactive GnRH and GnRH mRNA have also been observed (Gibbons *et al.*, 1975; Kelly *et al.*, 1991). Whether specific binding sites for GnRH are present in the guinea-pig placenta and sub-placenta requires further investigation.

There are a number of possibilities for future work in this field. With hindsight, the effects of the P2 purinoceptor antagonist, suramin, and the adenosine (A) receptor antagonist, 8-sulphophenyltheophylline, on PG outputs from the guinea-pig uterus could have been further investigated. Different concentrations of drug could have been used. In culture experiments with ATP, adenosine, suramin and 8-sulphophenyltheophylline, the effects of suramin and 8-sulphophenyltheophylline alone on PG outputs from the guinea-pig endometrium and myometrium would have proved useful. The use of more specific antagonist would also have been beneficial in determining which receptor subtypes were involved. Unfortunately, specific receptor antagonists were not available.

A specific PGHS-1 antagonist would have been useful to determine the involvement of PGHS-1 and PGHS-2 in PG production by the guinea-pig placenta and sub-placenta. Several PLA_2 enzymes have been identified in the human placenta (Freed *et al.*, 1997; Rice *et al.*, 1994; Buhl *et al.*, 1995). It would also have been useful to determine which PLA_2 enzymes are involved in PG production by the guinea-pig placenta and sub-placenta. There are PLA_2 antagonists and activators available but these are not specific. Northern and Western blotting techniques may have been

useful to demonstrate the presence of specific PGHS and PLA_2 activity.

The results presented in this thesis have demonstrated that the mechanisms involved in PG production by the mid-pregnant guinea-pig placenta and sub-placenta are complex. These include the synthesis and availability of PLA_2, PGHS and calcium (extracellular and intracellular). Different cells within the guinea-pig placenta may produce different PGs, and may have differentially regulated pathways for their production. There may also be expression of different phospholipase enzymes in the different cell types. Although PLA_2 type II appears to be the predominat phospholipase enzyme in the human placenta, several phospholipae enzymes have been observed in the placenta and may have a role to play in PG production. This may also be the case in the guinea-pig placenta and sub-placenta. Once released, arachidonic acid may form distinct pools which may only be available to specific PGHS enzymes (PGHS-1 or PGHS-2) for the production of specific PGs. This work has also shown that the guinea-pig placenta is a major source of PGs during pregnancy and that PG production by the guinea-pig placenta and sub-placenta may have an important role to play during gestation. The fact that PG output from the guinea-pig placenta, particularly $PGF_{2\alpha}$, increased as pregnancy proceeded suggests that PGs of placental origin may be required during pregnancy. Previous workers have suggested several functions for increased PG synthesis and output during pregnancy including regulation of uterine contractions, ripening of the cervix, regulation of the patency of the ductus arteriosus, luteolysis, fetal maturation and regulation of utero-placental haemodynamics (Moussard *et al.*, 1986; Hoedemaker *et al.*, 1991; Rice *et al.*, 1995). This study has looked at PG output from mid-pregnant guinea-pig placenta and sub-placenta. Therefore, it is unlikely that increased $PGF_{2\alpha}$

output from the guinea-pig placenta is involved in uterine contractions or ripening of the cervix. It remains to be established whether PGs produced by the day 29 guinea-pig placenta and sub-placenta are involved in the other processes mentioned.

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